

The Activity of Phosphorylase Kinase Revisited

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Abstract

Phosphorylase Kinase (PhK) is an essential regulatory enzyme in the glycogenolysis cascade. PhK is a large, 16-subunit enzyme complex with the subunit stoichiometry $(\alpha\beta\gamma\delta)_4$, and is subject to extensive regulation by small molecules and reversible phosphorylation. Because of its large mass of 1.3 MDa, only limited experimental tools are available to study the structure of PhK and elucidate the structural changes that occur within the complex concomitant with activation. Therefore, our understanding of the roles of PhK's subunits in regulating its kinase activity is woefully incomplete. The goal of this work was to revisit several aspects of PhK's activity and further explore the roles of the enzyme's individual subunits.

Chemical crosslinking was the principal technique used in these studies because it is well-suited to study large, multi-subunit complexes. Using chemical crosslinking, plus additional methods, three major discoveries are presented regarding PhK's activation and substrate recognition. First, zero-length oxidative crosslinking was used to selectively study conformational changes in the regulatory β subunits, which led to the formulation of a model for activation of PhK. The model proposes that modification of the N-terminus of β is key to activation of the catalytic γ subunit and to conformational changes in the β subunits, which likely cause global structural changes in the complex. Secondly, a two-step crosslinking approach revealed novel interactions between the regulatory α and β subunits and PhK's substrate, glycogen phosphorylase, establishing the first direct evidence of substrate binding sites on the regulatory subunits of the enzyme. Lastly, a temperature-dependent conformational change in the β and γ subunits was discovered to occur between the standard assay temperature of 30 °C and the physiological temperature of 40 °C. This temperature-dependent conformational change coincides with a surprising activation of PhK at physiological temperature.

Steady progress continues to be made in studying the PhK complex. While the picture is still far from complete, this work contributes important details regarding the α , β , and γ subunits

and their expanded roles in PhK's activation and substrate interaction. Further exploration of the structure and activity of PhK is necessary to fully understand its critical function in glycogenolysis.

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List of Abbreviations

AMP PNP, Adenylyl-imidodiphosphate
AMPK, AMP-dependent protein kinase
ANB-NOS, N-5-Azido-2-nitrobenzoyloxysuccinimide
DFDNB, 1,5-difluoro-2,4-dinitrobenzene
CaM, calmodulin
CaMK, calmodulin-dependent protein kinase
cGMP, cyclic guanosine monophosphate
cryoEM, cryo-electron microscopy
 γ CRD, C-terminal regulatory domain of the γ subunit
Glucose-1-P, Glucose-1-phosphate
Glucose-6-P, Glucose-6-phosphate
GMBS, N- γ -maleimidobutyl-oxysuccinimide ester
GP, glycogen phosphorylase
MS, mass spectrometry
PhK, phosphorylase kinase
PKA, cAMP-dependent protein kinase
PKC, protein kinase C
PKG1 α , cGMP-dependent protein kinase
PLP, pyridoxal phosphate
RT, room temperature
SIA, succinimidyl iodoacetate
UDPG, uridine diphosphate glucose

Chapter I: Introduction

Overview

Glycogen is a highly branched polysaccharide found in many organisms, including bacteria, fungi, and mammals. The polysaccharide is composed of D-glucose, linked by 1-4 α glycosidic bonds and 1-6 α glycosidic bonds that form branch points every 10-14 residues. Glycogen particles can contain up to 55,000 glucose molecules, providing a convenient way to store large amounts of cytosolic glucose (1). Large deposits of glycogen are found in the muscle and liver tissues of mammals, but smaller amounts of glycogen still play important roles in other tissues such as brain, skin, and cancer cells (2-4). In muscle, the fast release of glucose from glycogen helps sustain muscle contraction. In liver tissue, glycogen is catabolized between meals to maintain blood glucose levels. In the brain, glycogen is found in both neurons and astrocytes, and its metabolism is essential for memory formation and synaptic plasticity (5, 6). Emerging evidence also suggests glycogen metabolism is important for cancer cell growth and angiogenesis (7, 8). The function and regulation of glycogen throughout the body remains an active and fascinating field of study.

Studying the regulation of glycogen metabolism is essential to understanding the diverse roles glycogen plays throughout the body. Several enzymes are responsible for the coordinated synthesis and degradation of glycogen. Mutations in many of these enzymes, including their tissue specific isoforms, result in diseases characterized by dysfunctional glycogen storage and diverse phenotypes, such as exercise intolerance, growth retardation, and hepatomegaly, to name a few (1). Collectively, these diseases are known as glycogen storage diseases and affect thousands of people around the world (9, 10).

Degradation of glycogen will be the focus herein, with particular emphasis on two proteins critical for glycogen catabolism. In the cytosol, glucose-1-P is released from the non-

reducing ends of glycogen by glycogen phosphorylase (GP) (11). GP is regulated by allosteric effectors and reversible phosphorylation by phosphorylase kinase (PhK) (reviewed in (12)). Despite GP and PhK being two of the earliest enzymes characterized in the burgeoning field of biochemistry during the 1940s and 1950s, questions still surround GP and PhK, including their interaction with each other. Further investigation into GP and PhK continues to fill the gaps in our understanding, revealing important details on the strict cellular regulation of glycogen metabolism.

Glycogen Phosphorylase

GP is the enzyme responsible for the phosphorolysis of the non-reducing ends of glycogen in the cytosol, releasing glucose-1-P for the cell's use (Figure 1.1). GP is a member of the large glycosyl hydrolase family of enzymes and is sub-classified alongside glucosyltransferases that act on α -1,4 glycosidic linkages. In addition to the α -1,4 glycosidic linkages, glycogen also contains α -1,6 branch sites, therefore the complete degradation of glycogen also requires glycogen debranching enzyme, which removes the α -1,6 branch sites during catabolism (13). The sequence and structure of GP is highly conserved, and its homologs are found in all branches of life, from *E.coli* to humans (14). Three isoforms of GP (muscle, liver, and brain) are found in mammals, and they share 80-83% identity (15). Muscle GP is the most thoroughly studied isoform, and is well characterized in terms of its structure, activity, and regulation. GP was one of the earliest enzymes to be studied, and it remains an important focus of investigation today. Developing inhibitors of GP as a way of blocking glycogen degradation is a common therapeutic strategy for treating type 2 diabetes and cancer (7, 16).

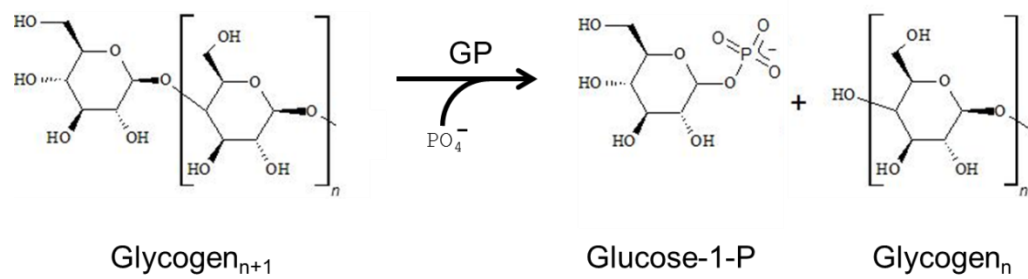


Figure 1.1 The GP reaction. Phosphorolysis of the non-reducing ends of glycogen to form glucose-1-P.

Structure

GP is a dimer of two identical subunits, each containing 843 amino acids (Figure 1.2)(17). The dimer of GP has two distinct faces, a convex side known as the regulatory face and a concave side known as the catalytic face as shown in Figure 1.2B (18). The substrate binding and active sites are located on the catalytic face of GP, buried in a ~ 5 Å channel that is partially blocked in the inactive state by the 280s loop (19). Also contained within the active site channel is a pyridoxal phosphate (PLP) group, an essential cofactor for GP, covalently bound to Lys680 (20, 21). Adjacent to the catalytic channel is the nucleotide inhibitor binding site (22). The substrate glycogen, in addition to binding to the active site, also binds a secondary site 30 Å away with a K_d of 1mM (23, 24). This second binding site, called the glycogen storage site, is also the site of tetramer formation between two GP dimers, as seen in the abundant x-ray crystal structures available for GP (19). GP readily crystallizes, suggesting that tetramer formation happens readily at high concentrations, however it is not known if GP tetramers exist *in vivo*.

The regulatory face of GP has two allosteric binding sites near the subunit interface. The first binding site, called the AMP binding site, binds IMP and glucose-6-P in addition to AMP, the main allosteric activator of GP (25, 26). The “new allosteric effector site” binds small molecule inhibitors of GP and is located 15 Å away from the AMP binding site (27). The 20 residues at the N-terminus of GP are flexible and disordered in the non-activated complex. The first 10 residues do not resolve in X-ray crystal structures and residues 11-20 are often poorly ordered (19). Additionally, the N-terminus is highly susceptible to proteolytic attack by trypsin or subtilisin, supporting the notion that this region is disordered (28). Importantly, contained within this flexible N-terminal tail of GP is the enzyme’s single phosphorylation site at Ser14 (29).

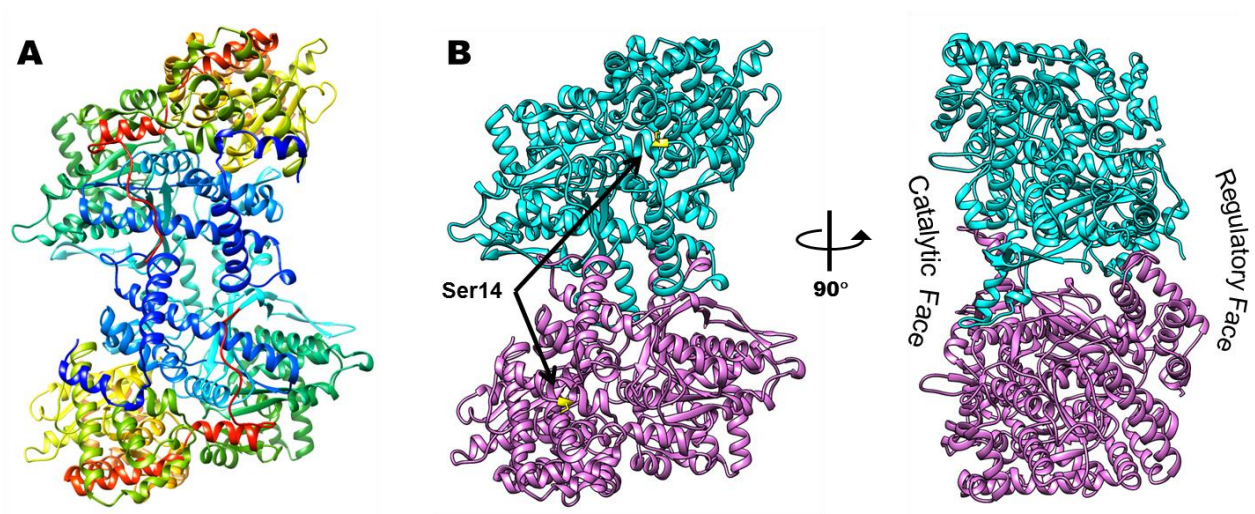


Figure 1.2 Structure of GP. (A) Ribbon diagram of GP, facing the regulatory side of the dimer. The N-termini are shown in blue and the C-termini are shown in red. GP was modeled with I-TASSER to generate a full-length model (the N-terminal 9 residues do not resolve in the crystal structures) and the homodimer was constructed with HADDOCK docking software. (B) Ribbon diagram of GP with one subunit in teal and the other in fuchsia. The left model views the regulatory face of GP and on the right model is a side-view of GP.

Catalytic Mechanism

GP carries out a rapid equilibrium, random bi-bi kinetic mechanism (30). Inorganic phosphate and glycogen bind in the active site channel, and inorganic phosphate undergoes a general acidic attack promoted by the 5' phosphate of PLP, forming a carbocation intermediate at the C1 position on the terminal glucosyl residue, cleaving the 1-4 glucosidic bond (31). The phosphate undergoes a nucleophilic attack on the carbocation, returning a proton to the PLP phosphate, completing the reaction. While glycogen degradation is GP's physiologically relevant activity, the reverse reaction (glycogen synthesis) proceeds readily in the presence of high concentrations of glucose-1-P. GP is commonly assayed in the direction of glycogen synthesis *in vitro* (32).

PLP is an integral part of GP's catalytic mechanism, but its role is unique from all other known PLP-containing enzymes. It is the phosphate group of PLP utilized in GP, and not the Schiff base that is used by all other known PLP-containing enzymes (33). Removal of the PLP in GP abolishes activity, but reduction of the Schiff base, which is attached to Lys680, has a minimal effect on activity (34). While PLP has an unconventional use in GP, it is found in every phosphorylase protein currently known, from yeast to humans.

Regulation

Phosphorylation of GP on Ser14 or binding AMP causes activation through significant tertiary structural changes at the subunit-subunit interface and quaternary changes that mediate the transition from the inactive T state to the active R state (Figure 1.3)(12). Although the bulk of GP (~60%) remains unchanged between the T and R states, the N-terminus undergoes significant rearrangement. Upon phosphorylation, the N-terminus rotates nearly 120°, switching from interacting with residues on the same subunit to making contacts across the subunit-

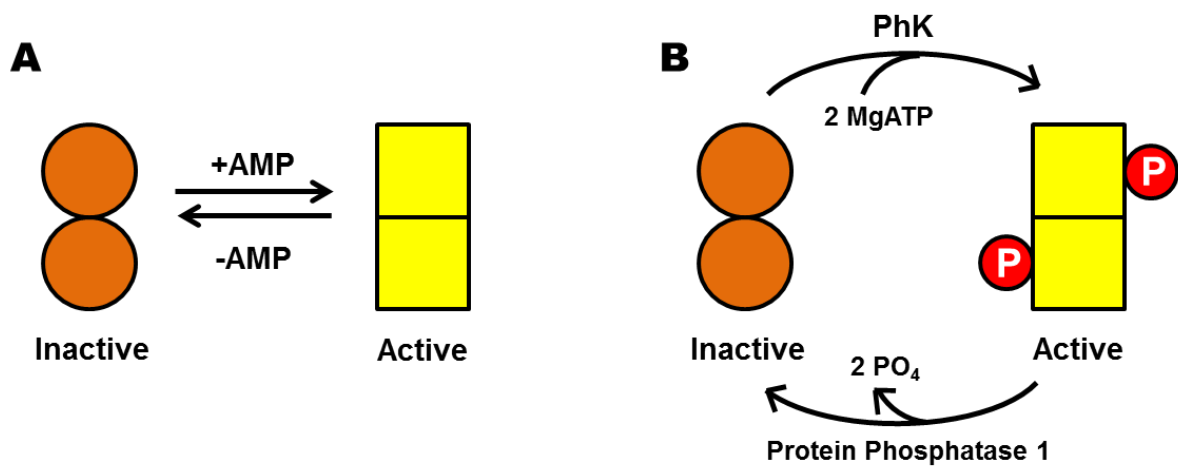


Figure 1.3 Regulation of GP. (A) AMP is an allosteric activator of GP. (B) Reversible phosphorylation regulates GP's activity and is controlled by the action of PhK and protein phosphatase 1.

subunit interface. This transition is driven by both electrostatic and hydrophobic interactions. The N-terminus has several basic residues that interact with an acidic patch on the surface of GP, but after phosphorylation, the phosphoserine repels this acidic environment to make contact with Arg69 and Arg43 near the interface. The hydrophobic residues surrounding phospho-Ser14, Ile13 and Val15, fit into nonpolar pockets at the interface. The rearrangement of the N-terminus also alters the AMP binding site located at the subunit interface. In the T state, AMP binding is facilitated solely through interactions with the phosphate group of AMP. In the phosphorylated R state, additional contacts are made with the ribose and adenine rings of AMP, accounting for the 100-fold increase in AMP affinity upon phosphorylation (19).

These changes near the subunit interface of GP cause a slight rotation of the two subunits (19). Accompanying these quaternary changes is movement of the tower helices on the catalytic face. The two tower helices, one from each subunit, cross one another at the interface, and in the T state their movement pulls the 280s loop from its location in the R state, exposing the active site to the solvent. GP is one of the first enzymes to have the signal transduction from effector binding to activation so thoroughly understood at the structural level.

Despite promoting similar conformational changes, AMP and phosphorylation do not have identical effects on GP. The differences between activation by AMP-binding and phosphorylation is even more pronounced among the different tissue isoforms of GP. In the case of the muscle isoform of GP, phosphorylated GP is active without AMP, but AMP binding can stimulate activity an additional 10-20% (35, 36). Conversely, AMP only weakly activates the liver isoform of GP, making phosphorylation the primary method of regulating GP activity in liver (37, 38).

Phosphorylation of GP

Reversible phosphorylation is a key mechanism for regulating GP activity. Despite the fact that many targets of protein phosphorylation are often targeted by multiple protein kinases, muscle GP is only known to be phosphorylated by one kinase, PhK, in response to the cAMP signal cascade. Phosphorylation by only one kinase is somewhat surprising because Ser14 on GP is likely to be solvent exposed, easily accessible, and the basic sequence surrounding Ser14 resembles the target sequences for multiple kinases, including the cAMP-dependent protein kinase (PKA) and protein kinase C. These results may indicate that Ser14 on GP is either not as accessible as previously thought or there is structure features near Ser14 that sterically block interactions with other kinases.

Phosphorylase Kinase

PhK is a large, regulatory kinase in the glycogenolysis cascade. The muscle isoform of PhK isolated from the fast-twitch psoas muscle of New Zealand White rabbits has been studied for over 60 years. PhK also has an important role in regulating glycogen metabolism in tissues other than muscle, such as brain and liver, where different isoforms of PhK are expressed (7, 39, 40). PhK is notable for several reasons: not only was it the first protein kinase to be isolated and characterized in 1955 by Edmond Fischer and Edwin Krebs, its immense size, structure, and complex regulation set it apart from all other known protein kinases (41).

Structure

The PhK holoenzyme has sixteen subunits, four copies of four distinct subunits: the catalytic subunit, γ , and three regulatory subunits, α , β , and δ (Table 1.1)(42). Altogether, the hexadecamer $(\alpha\beta\gamma\delta)_4$ has a mass of 1.3 MDa, making it nearly a third the size of the eukaryotic ribosome. Because of its immense size, PhK has eluded high resolution structural analysis, with the exception of the catalytic domain of γ and the small δ subunit. Decades of structural studies

Table 1.1 Features of the Subunits of Rabbit Skeletal Muscle PhK

Subunit	Role	Size	Genes	Regulation or Modification	High Resolution Structures
γ	Catalytic	386 aa 44,673 Da	PHKG1 (Muscle) PHKG2 (Liver/Testis)	Inhibited by α , δ , and β	Residues 1-300 (PBD ID 1PHK)
α	Regulatory	1237 aa 138,422 Da	PHKA1 (Muscle) PHKA2 (Liver)	Phosphorylation Farnesylation	Computational Models
β	Regulatory	1092 aa 125,205 Da	PHKB	Phosphorylation ADP Binding Farnesylation	Computational Models
δ	Regulatory	148 aa 16,680 Da	CALM1 CALM2 CALM3	Ca ²⁺ Binding	Crystal Structures for Free CaM/ δ

utilizing cryo-electron microscopy (cryoEM), chemical crosslinking, and top-down mass spectrometry (MS), have revealed the likely arrangement of the subunits within the complex and how their interactions change upon activation (Figure 1.4)(43-46). The PhK hexadecamer is composed of two octamers stacked in D2 symmetry, creating a bilobal structure connected by four bridges (45). The regulatory β subunits form the central bridge-containing core of the protein (47). Around the β core are arrayed trimers of α , δ , and γ . The α , δ , and γ subunits form the distal portion of the lobes and can be expressed as soluble, stable trimers (48). Crosslinking and top-down MS studies suggest that every subunit within PhK makes physical contact with the remaining three types of subunits, creating a complex structural network within the holoenzyme (46).

γ Subunit

The catalytic site of PhK is located on the γ subunit, which has a mass of 45 kDa and is separated into two domains: a catalytic domain and a regulatory domain (49). The N-terminal catalytic domain spans approximately 300 residues and has a typical protein kinase sequence and corresponding bilobal structure. The N-terminal lobe of the kinase is composed of predominately β sheets, connected to the larger C-terminal lobe which is composed mostly of α helices. The hinge between these two lobes creates the conserved ATP binding site and the site of catalysis (50). A truncated form of γ containing only the catalytic domain has been successfully crystallized with and without a bound peptide substrate (51). The γ structures have revealed important information regarding substrate binding to the PhK active site, including the importance of basic residues surrounding the phosphorylation site for interactions with residues on γ (51).

The C-terminal regulatory domain of γ (γ CRD) contains two high affinity calmodulin (CaM) binding sites (52), one of which directly interacts with the δ subunit of PhK, which is CaM

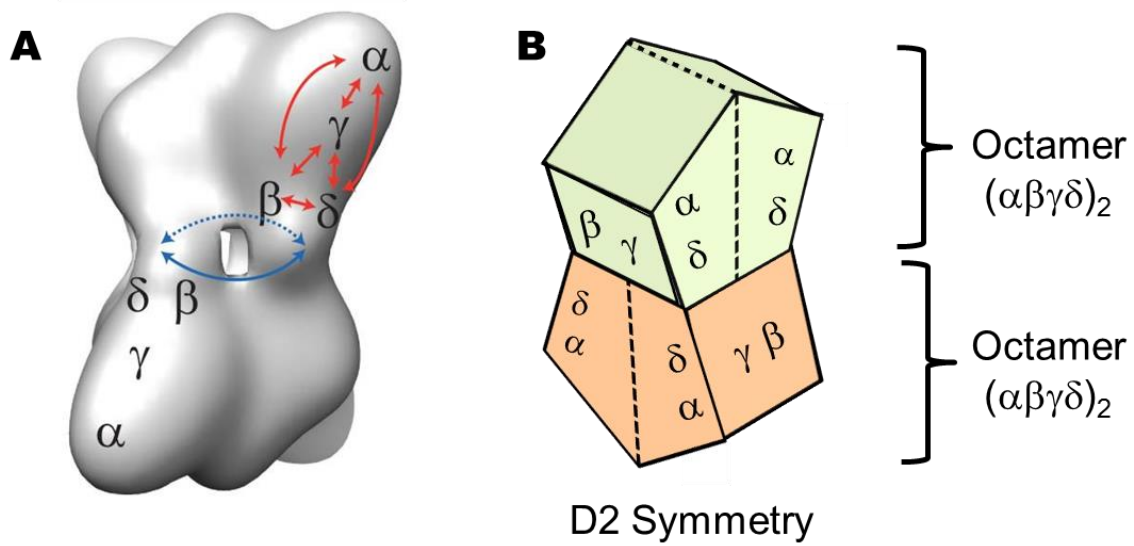


Figure 1.4 PhK Structure. (A) Cryo-EM reconstruction of the non-activated PhK molecule with the approximate locations of the four subunits in two of the four protomers (used with permission from (46)). (B) Schematic drawing of the PhK hexadecamer to illustrate the D2 symmetry of the complex.

(53). The γ CRD has also been shown to be proximal to the other two regulatory subunits, α and β (54, 55). Crystal structures are not available for full-length γ ; however computational modeling using the threading program I-TASSER suggest the structure of the γ CRD is similar to the regulatory domains of some other CaM-dependent protein kinases (CaMK), such as the death associated protein kinase (56).

δ Subunit

Although PhK is a member of the CaMK family (42), it is an atypical member because in the absence of Ca^{2+} , δ /CaM remains bound to the complex, unlike other CaM kinases that dissociate from CaM without Ca^{2+} present (57, 58). The integral nature of CaM in PhK may be due to an interaction between the third EF hand of δ and the γ subunit (53). In addition to anchoring δ regardless of Ca^{2+} concentrations, the interaction with the EF hand likely explains why PhK only binds 3 out of the 4 expected Ca^{2+} ions for CaM (59). Dimers of γ and δ , which have been successfully isolated from the holoenzyme and expressed recombinantly, carry out Ca^{2+} dependent phosphorylation of substrates (60, 61). Isolated γ also binds free CaM with high affinity ($K_d = 25$ nM) (62).

α and β Subunits

Over 80% of the mass of PhK is composed of the large, regulatory α and β subunits. Homologs of one another, α and β have masses of 138 kDa and 125 kDa, respectively (63). Based on sequence similarity, both subunits contain calcineurin B-like domains in their C-termini and glucoamylase-like domains resembling those of the glycosyl hydrolase family 15 (GH15) in their N-termini (64, 65). In the glucoamylase-like domain of the α subunit, the two catalytic glutamate residues necessary for GH15 hydrolysis activity are present (64). Additionally, α binds glycogen and a small amount of glycogen hydrolysis has been observed (66). Both subunits are the target of effectors and reversible phosphorylation. The phosphorylation sites

are located in unique regions of the homologous subunits. Phosphorylation of the unique N-terminus of β is a key regulatory event in PhK activation, and is targeted by autophosphorylation and PKA phosphorylation (63). The α subunit contains a short multiphosphorylation sequence in its C-terminus (67).

Catalytic Mechanism

PhK catalyzes the transfer of the γ phosphoryl group of ATP to a hydroxyl group on a target protein (Figure 1.5). Thus far, all known protein kinases proceed via a sequential mechanism, with the phosphate group being transferred directly from ATP to the substrate, as opposed to forming a phospho-enzyme intermediate (68). Whether the substrate binding is ordered or random appears to be dependent on the individual kinase. In the case of PKA, binding is random, but with a bias towards ATP binding before the protein substrate (50). For PhK, a random mechanism has been reported, suggesting that ATP and GP may bind in either order (69). Kinetic mechanisms appear to differ from one kinase to another, ruling out the possibility for a universal mechanism to describe all kinases. Additionally, several aspects of the kinase reaction have still not been resolved, including the reaction intermediate (associative vs dissociative) and the role of several universally conserved residues in the kinase active site (50, 68). Given several conserved charged residues in the active site, some postulated that kinases utilize general acid-base catalysis to abstract a proton from the target hydroxyl group; however, most of the evidence from mutational studies, computational modeling, and kinetics do not support this hypothesis (50). The role of the conserved active site residues may instead be to orient the substrates and stabilization of the reaction intermediates. The catalytic Asp149 in PhK is absolutely conserved, and from crystal structures, interacts with the essential Mg^{2+} that coordinates the β and γ phosphates of ATP, likely orienting them for phospho-transfer (70).

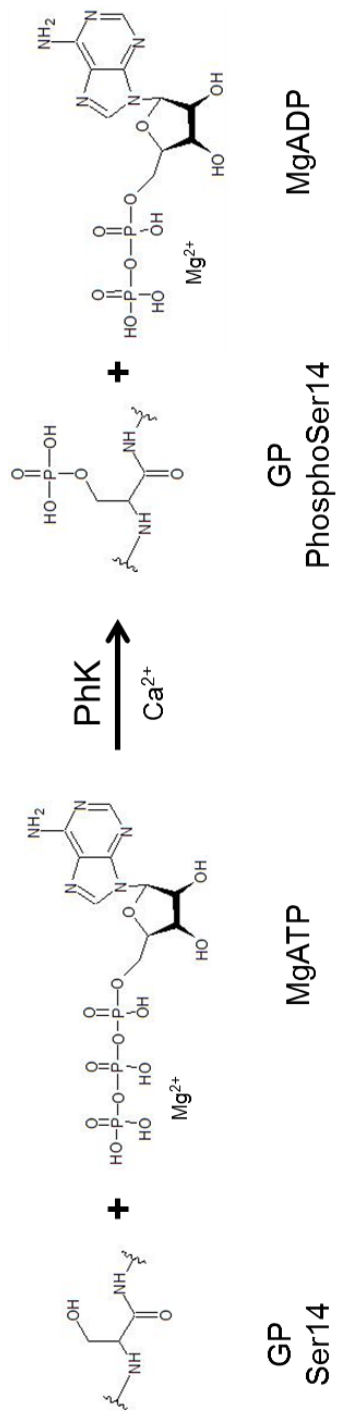


Figure 1.5 PhK Reaction. Phosphoryl-transfer of the γ -phosphate group of ATP to Ser14 on GP.

Kinetic studies on PhK have been relatively sparse and generally disagree with one another. There are several reasons for this (see below), but one consequence is a considerable range of reported kinetic constants. Previous reports support a rapid equilibrium, random bi-bi mechanism for PhK (69, 71). A more recent report by Skamnaki *et al.* (70) argues against a rapid equilibrium, but the reported Michaelis constant for GP is still similar to those reported previously (5 μM compared to 20 μM) (Table 1.2). With full length GP as substrate, the reported K_m values for phospho-activated PhK range from 5.5 μM to 125 μM (72, 73).

Two reasons for the discrepancies in reported kinetic values are 1) PhK's non-linear activity and 2) differences in assay conditions between studies. Non-activated PhK has a lag in activity (enzymatic hysteresis) (74), precluding analysis using Michaelis-Menten kinetics, which requires linear initial activity. Thus, most kinetic studies with PhK have been performed on the activated complex, which has no hysteretic behavior. The lag was initially thought to be due to auto-activation of PhK by phosphorylation or complex dissociation, but later results suggest that the hysteresis is likely due to a slow conformational change caused by binding divalent cations (75). Whether activation of PhK changes its V_{max} or K_m values is not known because of the hysteretic behavior of non-activated PhK. Krebs and Fischer postulated in 1964 (74) that a change in K_m for GP gives rise to PhK activation, but this has been challenged several times. A K_m effect is also inconsistent with the supersaturating amounts of GP present in muscle cells, making K_m changes practically irrelevant *in vivo* (76, 77). A later publication by Newsholme (73), suggest a V_{max} effect associated with PhK activation.

Experiments performed with PhK, kinetic experiments in particular, vary considerably in their design and composition from one report to another. Some experiments utilize the hexadecameric PhK complex, others truncated γ , with either full length GP or peptide substrates. PhK's activity is sensitive to multiple factors that often vary between studies including the buffer,

Table 1.2 K_m and K_d Values Reported for PhK

Reference	K_m (GP)	Reported a Rapid Equilibrium?	K_d	Methodology
Tabatabai and Graves (69)	20 μ M	Yes	36 μ M	Initial Velocity Kinetics
Skamnaki <i>et al</i> (70)	ND	No	5 μ M	Viscosity Experiments
Xu (110)	ND	ND	2-40 nM	ELISA

pH, Mg^{2+} concentration, salt concentration and temperature (78-81). These differences likely contribute to some of the differences among reports.

Substrates

PhK catalyzes a bimolecular reaction between MgATP and a protein substrate and the products of this reaction are MgADP and a phosphorylated protein. MgATP has a K_m value of 0.20mM at pH 7.5 for activated PhK (69). In the case of PKA, ADP release is slow and the rate-limiting step (82), however it is not clear if the rate limiting step for PhK is also ADP/product release.

GP is considered to be PhK's primary target *in vivo*, however, *in vitro* PhK phosphorylates several other protein targets. Glycogen synthase is phosphorylated by PhK nearly as well as GP, but because synthase is the target of multiple protein kinases, the biologically significant PhK phosphorylation is unknown (83). Other PhK substrates include the regulatory α and β subunits of PhK (via autophosphorylation), myosin light chain, troponin, tau, neurogranin, and water (ATPase activity) (84-88). These additional substrates of PhK are phosphorylated at a much slower rate than GP or glycogen synthase and paradoxically, ATP-analog affinity labels cause variable rates of inactivation depending on which protein substrate is being used (89). Additionally, peptide substrates stimulate auto-phosphorylation of PhK, even at high peptide concentrations, when one would expect to see competitive inhibition (90). These results, along with other seemingly paradoxical findings, led to an early hypothesis that PhK had two distinct active sites (reviewed in (91)). Today, we know there is only the one kinase active site on the γ subunit, but the explanation for the dissimilar behavior towards two distinct groups of protein substrates remains unresolved given that each protomer in a tetrahedral complex will be identical.

The importance of the amino acid sequence surrounding Ser14 on GP for PhK phosphorylation has been studied in great detail, usually with synthetic peptides. One key characteristic of the N-terminus of GP is the four basic residues surrounding Ser14, making PhK a basophilic kinase. Peptide studies have revealed a critical role for Arg16, in that charge removal or reversal of Arg16 cause a large reduction in PhK phosphorylation of the peptide (72). Peptides with an Ala substitution for Arg16 are equally good substrates for PKA and PhK (92). Furthermore, although full length wild-type GP is not a substrate for PKA, an R16A GP mutant can be slowly phosphorylated by PKA (93). This suggests that Arg16 plays an important role in selecting for phosphorylation by PhK, but not PKA. Mutation of any of the residues proximal to Ser14 typically produced a peptide that was a poorer substrate than the wild-type peptide (72). The corresponding point mutations of full length GP were also poorer substrates than wild-type, but the difference was less pronounced than observed with the peptide mutants (93).

Peptide studies based on known protein substrates are valuable but inherently biased by the existing sequences of known substrates. To study the unbiased, optimal target sequences for kinases, the Cantley group designed an oriented peptide library screening approach (94). The optimized sequence for PhK from the peptide library had Phe and Met residues flanking the phosphorylatable serine and did not resemble the phosphorylation site on GP. Although this unusual sequence may be optimized for PhK phosphorylation, it lacks the context of the intact GP molecule, which may assist with substrate binding and phosphorylation. What the optimized peptide does reveal is the necessary flexibility near the active site of γ to accommodate significantly different sequences, which is further supported by the observation that PhK can phosphorylate the CaM binding peptide mellitin (95). When the sequence of mellitin is compared to the GP sequence, it is only when mellitin is viewed from the C- to N-terminus that the sequences align, raising the possibility that γ may be able to bind a substrate sequence in the reverse orientation.

Regulation

PhK is subject to a high degree of regulation, from small molecule effectors to reversible phosphorylation (reviewed in (96)). Given PhK's large size, one might question why a single-function enzyme like PhK requires and has evolved such a complex structure. One possibility is that PhK acts as a signaling hub for regulating glycogen degradation, evolving multiple regulatory sites to respond to diverse cell signaling pathways (hormonal, neuronal, and metabolic) (Figure 1.6). Alternatively, PhK may have additional functions or protein targets that are currently unknown, potentially in tissues other than muscle.

A truncated form of γ that contains only the catalytic domain, either expressed recombinantly or proteolytically derived from muscle PhK, is constitutively active and Ca^{2+} /CaM-independent (97, 98). Unlike other kinases that require phosphorylation of the activation loop near the active site for activation, Glu182 on the activation loop of PhK appears to obviate the need for phosphorylation (51). In contrast, the homologous residue on PKA, Thr197, requires phosphorylation in order to become active (51). Furthermore, truncated γ has the same specific activity as maximally activated PhK, arguing for auto-inhibition of γ in the non-activated hexadecameric complex. The role of the regulatory subunits and domains of PhK, which account for 90% of the enzyme's mass, inhibit the activity of γ in the holoenzyme through quaternary constraints (99, 100). A common mechanism in kinases is for an auto-inhibitory sequence to block enzyme activity and PhK contains several potential auto-inhibitory segments: two sequences in the γ CRD and one in the β subunit that inhibit γ as peptides (52, 101). All three regulatory subunits are

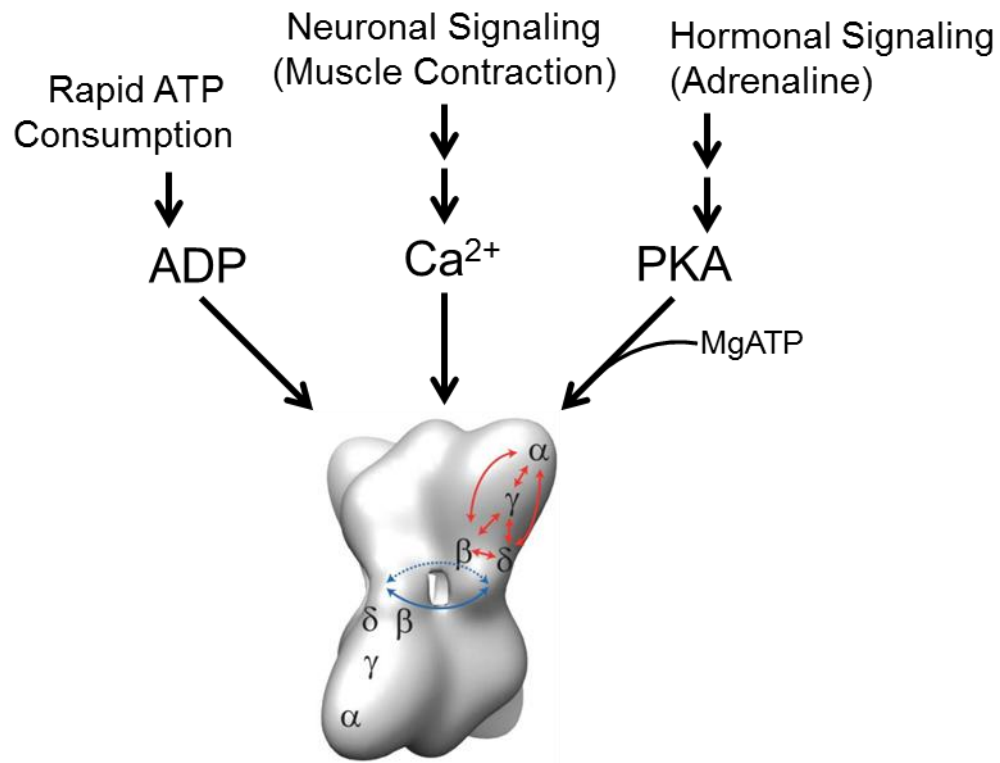


Figure 1.6 PhK Regulation. Muscle PhK is regulated by several different signaling pathways, including neuronal, hormonal, and metabolic signals.

proximal to the γ CRD, which reportedly acts as an allosteric switch mediating signals from the regulatory subunits to the nearby active site (55).

Ca^{2+} is absolutely required for PhK activity and Ca^{2+} binding is mediated by the δ subunit of PhK (42). Ca^{2+} has also been shown to cause global conformational changes to the PhK complex (44). In addition to Ca^{2+} , other divalent cations also regulate PhK's activity. Mg^{2+} and Mn^{2+} bind at the active site of γ and at a second binding site. Free Mg^{2+} binding to the second site on PhK stimulates activity, whereas Mn^{2+} inhibits PhK (102). While Ca^{2+} binding is clearly mediated by δ , Mg^{2+} and Mn^{2+} likely exert their opposing effects through binding the γ subunit (102). Mg^{2+} is also known to cause considerable physiochemical changes to the PhK holoenzyme (80).

The PhK α and β subunits are the targets of phosphorylation by PKA and by PhK itself. Phosphorylation of the β subunits is key for activation, with the selective dephosphorylation of β by protein phosphatase 1 leading to inactivation of the enzyme, regardless of the amount of phosphate incorporated into the α subunit (103, 104). Phosphorylation of β by PKA happens rapidly, incorporating 1 mol phosphate per mol of β while phosphorylation of α rises gradually, eventually incorporating multiple phosphates per mol of α (104). The phosphorylation of α causes an additional increase in PhK activity (104). Autophosphorylation also leads to phosphorylation of β and α , but whether the phosphorylation sites are the same as those with PKA phosphorylation or autophosphorylation occurs *in vivo* is unclear.

In addition to phosphorylation, PhK is activated at pH values above 7.0, reaching a maximum at pH 8.2 (74). The ratio of PhK's GP phosphorylation activity at pH 6.8 (non-activated) and pH 8.2 (fully-activated) has been the standard method for reporting PhK activation for over half a century.

Several additional effectors have been reported to regulate PhK's activity *in vitro*. ADP is an allosteric activator of PhK and has two distinct binding sites on the complex: a high and low affinity site ($K_d = 2 \mu\text{M}$ and $17 \mu\text{M}$) (79). The allosteric activation site of ADP binding is very likely on the β subunits, but the location of the second ADP binding site is less certain (79). In addition to ADP, AMP, FAD, and glycogen bind and activate PhK *in vitro* (105-107).

Lastly, PhK is activated *in vitro* by limited proteolysis by multiple proteases. The α subunits of PhK are particularly susceptible to digestion, likely due to their exposed location near the lobe tips of PhK (108). More recently, PhK has been shown to be the target of caspase 3 cleavage (109). The relevance of proteolytic cleavage of PhK *in vivo* is not known.

Activation of PhK by Ca^{2+} binding, phosphorylation, and/or alkaline pH is associated with several conformational changes to the complex. Many of the structural changes appear to be accompanied by changes in the β subunits. The bridges, composed of the β subunits, appear to shift upon activation accompanying global changes in the lobes (44). Contacts among the β subunits appear to strengthen in the activated complex while interactions between the other three subunits appear to weaken (46). Activation of PhK and the corresponding conformational changes is clearly a complex process and one that will require further investigation.

Interaction Between GP and PhK

Although there is still much we do not know about PhK and GP independently, we know even less about their interactions with each another. For example, we still do not know how tightly GP binds to PhK, how many GP molecules can bind to a single PhK holoenzyme, or whether GP binds to any regulatory regions of PhK.

Studies on the interactions between PhK and GP are few in number. The sizes of PhK, GP, and the complex they form when bound together ($\geq 1.5 \text{ MDa}$) are not amenable to many traditional binding techniques. Previous attempts to use fluorescence anisotropy, capillary

electrophoresis, light scattering, and Hummel-Dreyer chromatography to determine a dissociation constant for the PhK-GP complex were not successful (110). One approach utilizing a modified ELISA was successful in determining the apparent dissociation constant for the PhK-GP complex and produced unexpected results (111). GP was detected to tightly bind PhK with nanomolar affinity (39.6 nM) that was enhanced 30-fold by Ca^{2+} , Mg^{2+} , and Mn^{2+} (2.9-5.2 nM). Importantly, these results from direct ELISAs agreed with the dissociation constants from competition ELISAs, which monitored the PhK-GP interaction in solution.

This tight nanomolar affinity was unexpected considering published kinetic studies. Although it is usually an over-simplification to equate a Michaelis constant with a K_d , if an enzyme undergoes a rapid equilibrium bi-bi kinetic mechanism, the K_m can equal K_d . From an early kinetic study of PhK that reported a rapid-equilibrium for PhK, the apparent K_d for GP was 36 μM (69). A later kinetic study by Skamnaki *et al.* (70), defined individual rate constants for the PhK reaction using viscosity experiments and although the authors found that PhK did not undergo a rapid-equilibrium with substrates, the K_d calculated directly from their rate constants was 5 μM . The apparent K_d values based on these kinetic studies (5 and 36 μM) are 1000-fold higher than the K_d values from the ELISAs (Table 1.2)(69, 70). The reason for the 1000-fold discrepancy between the binding affinity from the ELISA study and the kinetic studies is not known. Application of new methods to measure PhK and GP binding will hopefully resolve this discrepancy in the future.

Details on the physical binding sites on PhK and GP for each other have not been well defined. The interactions between an enzyme and its protein substrate can be subdivided into two kinds of interactions. The first is the physical contacts made at the active site between the atoms or bonds to be modified and the surrounding residues that help position or stabilize the reaction. The other type of interactions are all those physically separated from the active site. These distal interactions can serve important functions in substrate recognition and binding

(112). Distal interactions are generally specific to a given kinase-substrate pair and are less well studied than active site interactions. For most kinases, including PhK, consensus sequences that encompass the active site interactions have been identified after years of work with peptide substrates but little is known about distal contact sites and their significance.

The ELISA studies discussed above also revealed important information about potential distal contact sites between PhK and GP. GP', which lacks the N-terminal 16-18 residues, binds to PhK with nanomolar affinity, but does not bind to isolated γ (113). These results suggest PhK has distal contact sites for GP (apart from the obligatory interaction between the N-terminus of GP and γ) and that these sites may be responsible for the nanomolar affinity binding detected in the ELISA experiments. It is not known where the GP docking site(s) reside on PhK, but yeast two-hybrid experiments have suggested that the α subunit of PhK may bind to GP and isolated δ /CaM has been shown to bind an N-terminal fragment of GP (114, 115). A previous report utilizing fluorescence polarization postulated that two molecules of the GP dimer may bind to PhK, however, given the short half-life of the fluorescence label utilized, it is unclear whether these results are accurate (116). Low resolution cryoEM experiments have been performed on PhK \pm GP, but sample heterogeneity, uncertainty in the percent GP bound, and unintended proteolysis of the PhK molecule complicate the interpretation of these results (117). Even so, with GP bound, the density of the lobes appear to increase, suggesting that GP is binding to the distal portions of the lobes which are composed of α , γ , and δ as opposed to the central bridge region of PhK that is composed of the β subunits.

In muscle cells, both GP and PhK are likely associated with the glycogen particle (118). Andreeva *et al.* (119) proposed that glycogen, PhK, and GP form a ternary complex, in which both enzymes are bound to each other and glycogen, PhK through its α subunits and GP

through its storage sites. The authors suggest that binding to glycogen may promote the interaction between PhK and GP.

Despite the clear challenges facing researchers studying PhK and GP together, their interaction is absolutely essential to the cellular degradation of glycogen and warrants continued effort to understand their complex interaction.

Research Aims

In the six decades since its discovery in 1955, a great deal has been discovered regarding the structure, regulation, and activity of PhK. Nevertheless, PhK remains an important target of investigation, especially given the emerging roles for glycogen metabolism in diseases like cancer and diabetes (3, 4, 16). Furthermore, as techniques in structural biology continue to improve and expand, working with difficult, complex structures like PhK becomes more feasible. Several key questions still remain regarding multiple aspects of PhK's activity. The work presented herein seeks to address three of these outstanding questions and is outlined below.

- 1) How is phosphorylation of PhK associated with activation of γ and conformational changes in the β subunits?
- 2) What regulatory subunits of PhK are involved in GP binding?
- 3) How are PhK's activity, conformation, and interaction with GP altered when investigated under more physiological conditions?

In addressing these questions, valuable insight into the activity of PhK and its essential interaction with its substrate GP will be gained.

Chapter II: A model for activation of the hexadecameric phosphorylase kinase complex deduced from zero-length oxidative crosslinking

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Introduction

Phosphorylase kinase (PhK) is a key regulatory enzyme in the glycogenolysis cascade, catalyzing the Ca^{2+} -dependent phosphorylation and activation of glycogen phosphorylase in response to neural, hormonal, and metabolic signals (96). PhK, a member of the Ca^{2+} /CaM-dependent protein kinase family, is a 1.3 MDa hexadecamer composed of 4 copies of 4 subunits, α , β , γ and δ . In non-activated PhK the regulatory α , β and δ subunits exert quaternary constraint on the catalytic γ subunit, inhibiting its kinase activity (59, 100). Release of this constraint, *i.e.*, activation, is achieved through phosphorylation by cAMP-dependent protein kinase, principally within the N-terminus of the β subunit (104).

Because of its large size and heterogeneous post-translational modifications, a high resolution structure is not available for PhK. Low to moderate resolution methods for structure determination, such as electron microscopy, small-angle X-ray scattering, native MS, chemical crosslinking and partial proteolysis, have illuminated approximate subunit locations and interactions in the PhK complex (45-47). The large, regulatory β subunits have been localized to the core of the complex, forming four central bridges that connect two octameric lobes [*i.e.*, $2(\alpha\beta\gamma\delta)_2$] (47).

One goal of our work has been to elucidate the structural mechanism for activation of the PhK complex by phosphorylation. Proximal regions of the β and γ subunits have been shown to be structurally and functionally coupled, in that activators of PhK, including phosphorylation, increase solvent accessibility of epitopes on both subunits (120). Moreover, phosphorylation promotes global conformational changes in PhK that alter interactions among the β subunits

within its β_4 core and between its β and γ subunits (46, 122). A large body of evidence points to the N-terminus of β as an important regulatory region in the structural and functional coupling of β and γ . This region of β contains two phosphorylatable serines, 11 and 26, whose phosphorylation is associated with activation of the kinase (55, 63, 104, 123). Two approaches have shown that within the PhK complex the N-terminus of β is proximal to both the C-terminal regulatory domain of γ (γ CRD) and to its active site. First, Lys303 within the γ CRD is crosslinked to Arg18 at the N-terminus of β by N-[γ -maleimidobutyroxy]succinimide ester (GMBS) (55). Second, autophosphorylation at the N-terminus of β occurs intramolecularly (124), indicating that this region of β can bind directly to the active site of γ . The interaction of the N-terminus of β with the γ subunit has also been studied using a synthetic peptide corresponding to the N-terminal 22 residues of β (referred to herein as the N β peptide), which contains the phosphorylatable Ser11. N β peptide inhibited phospho-activated PhK; and when present during crosslinking with GMBS, N β not only blocked crosslinking of β to γ , but was itself crosslinked to the same Lys in the γ CRD as was the β subunit (55). These results indicate that the N β peptide is a true mimetic of the N-terminus of β , competing for the same binding site(s) within the PhK complex. Phosphorylation of Ser11 on N β blocked the peptide's ability to inhibit phospho-activated PhK (55).

Given that previous work suggested that the N-terminal region of β influenced both β - β and β - γ interactions in the PhK complex, our goal in this current study was to directly examine the effect on β self-association of disruption of the β - γ interaction by the N β peptide. This approach required a highly selective crosslinker capable of forming β - β dimers in reasonable amounts within the intact PhK hexadecamer. Oxidative crosslinking, an emerging tool for studying protein-protein interactions (125), was the technique that was used to capture the β - β dimers. Oxidation of susceptible side chains can lead to reactive centers that are readily

attacked by nearby nucleophiles, creating zero-length crosslinks within a protein (126). The general oxidizing agent periodate was selected for this study because it was found to be highly selective and efficient in crosslinking PhK.

Results

Characterization of the Products of Periodate Crosslinking

Periodate oxidation of PhK led to formation of three new species: a high molecular mass (~180 kDa) crosslinked conjugate running slower than the heaviest subunit of PhK, and two low molecular mass species migrating slightly slower than the γ subunit (Figure 2.1). The crosslinked heavy conjugate was determined to be intramolecular, as opposed to intermolecular (*i.e.*, formed within one PhK hexadecamer as opposed to between two), based on co-elution of the crosslinked PhK with the native enzyme on a size exclusion column (data not shown). Because common buffer components are potentially susceptible to oxidation, we performed control reactions with alternative buffers and without sucrose and found that periodate still formed the same reaction products to the same extent, indicating that the modification of PhK was directly caused by periodate as opposed to a byproduct of periodate-oxidized buffer components.

The compositions of the three new species were determined using densitometry, apparent molecular masses, Western blot analyses, and N-terminal sequencing. The time-course of crosslinking shows a time-dependent loss of the monomeric β and γ subunits, while the heavy crosslinked species appears at a rate similar to the loss of β before approaching a plateau at 15 min (Figure 2.1). The α and δ subunits, on the other hand, show no change in density over time, and

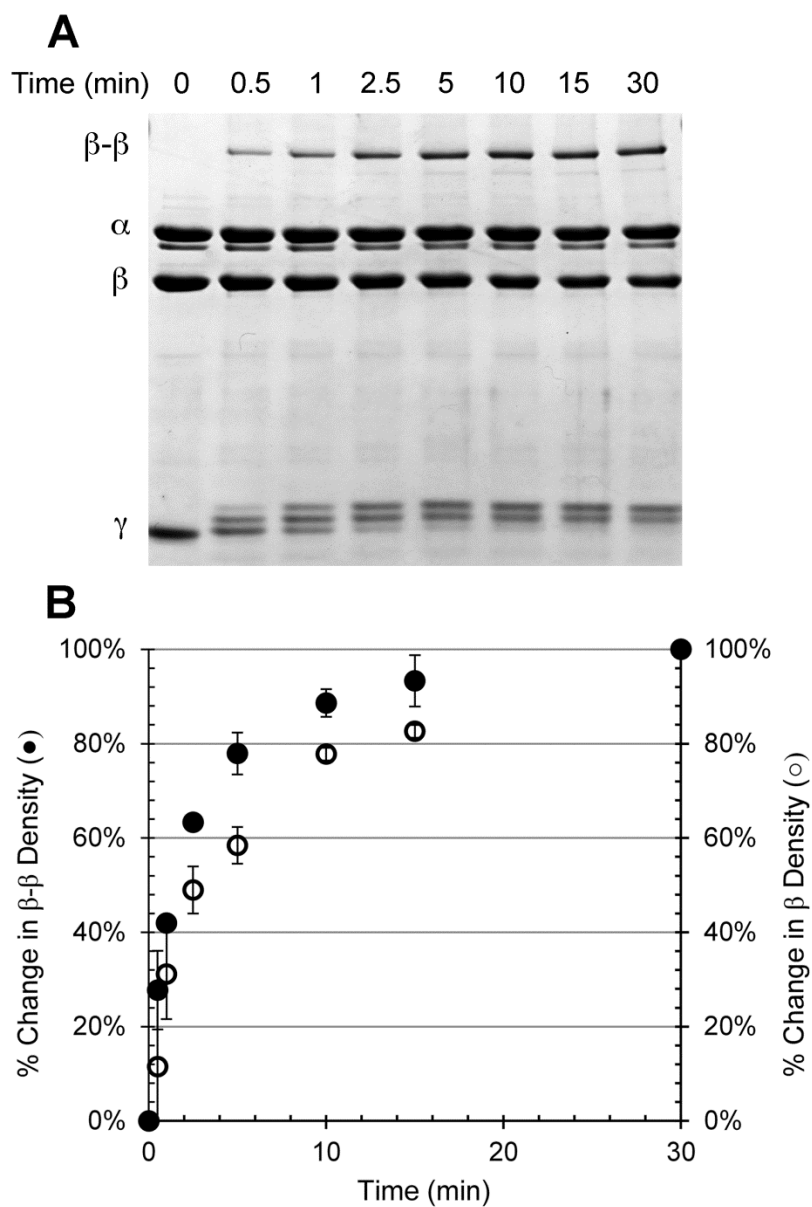


Figure 2.1 Time-dependent modification of PhK by periodate. (A) Coomassie stained 6-18% SDS-PAGE, time course of crosslinking. (B) Percent change in the densities over time of the crosslinked β - β (●) and monomeric β (○) bands. Error bars represent standard deviation of triplicate samples.

thus manifest no evidence of participation in the periodate crosslinking. The apparent molecular mass of the heavy conjugate (180 kDa) is consistent with a β -dimer (theoretical 220 kDa) or a $\beta\gamma\gamma$ -trimer (theoretical 190 kDa). It should be noted, however, that crosslinked proteins may display a smaller apparent mass than the simple sum of their individual components due either to crosslinking in central regions of the polypeptides or to additional intra-polypeptide chain crosslinking, both leading to smaller effective Stokes radii and thus faster migration. Western blots of the periodate-modified enzyme, using subunit-specific monoclonal antibodies for α , β , and γ (Figure 2.2), revealed the heavy conjugate to be composed of only β , consistent with a β -dimer. The three new species were N-terminally sequenced, and γ was detected with high confidence in the two lighter bands only. The β subunit is acetylated at its N-terminus (63) and thus was not detected. To further confirm that the heavy conjugate is a β -dimer, β was phosphorylated to a known extent by PKA and the resultant PhK crosslinked with periodate. If it is a β -dimer, the specific radioactivity of the crosslinked product should be equivalent to the specific radioactivity of the monomeric β . If instead the heavy conjugate is composed of β and γ , its specific radioactivity would be expected to be lower than monomeric β . The specific radioactivities of the heavy conjugate and the β monomer corresponded, indicating that the heavy conjugate is composed entirely of β (data not shown). We therefore concluded that the heavy molecular mass species is a β -dimer.

The cause of the plateau in β - β crosslinking after approximately 15 min is unknown. Although virtually all of the native γ is modified, over 50% of the monomeric β remains (Figure 2.1). Importantly, addition of more periodate after crosslinking reached its plateau did not cause more β -dimer to form, indicating that the plateau is not due to exhaustion of the periodate but instead to an inability of the remaining β to be crosslinked. This could be explained by the presence of a subpopulation of β that is exclusively targeted by periodate, or alternatively

oxidation of β over time leading to modification of the same amino acid residues that would have otherwise participated in crosslinking. We think that this second possibility is highly possible, given that at least 8 different amino acids can be oxidized by periodate (see Discussion). Oxidation of any of these residues would be occurring simultaneously with, and could compete with, a crosslinking reaction by forming an oxidized dead-end side chain incapable of crosslinking.

The two low molecular mass species formed by periodate oxidation appear to be composed entirely of γ (Figure 2.2). The apparent molecular masses of these two species are 43 and 45 kDa, or two and 4 kDa heavier than native γ , respectively. Although the increase in apparent mass is consistent with conjugation with a small peptide, we found no evidence for this. The oxidative modification of γ likely causes the anomalous gel migration, as has been seen with oxidation of superoxide dismutase and is analogous to the altered migration frequently observed with other proteins after their phosphorylation (127, 128).

PhK Effectors and β Crosslinking

Several small molecules, such as divalent cations and nucleotides, bind to PhK and modulate its activity by bringing about conformational changes in the enzyme complex. Nucleoside diphosphates, in particular, are hypothesized to target the β subunit and promote conformational changes within the β -core of the enzyme (79). We therefore crosslinked PhK with periodate in the presence of four known effectors: Mg^{2+} , Ca^{2+} , deoxyADP and deoxyGDP. Deoxyribonucleotides were used instead of ribonucleotides to avoid oxidative cleavage of the ribose ring by periodate. Removal of the 3' hydroxyl group from ADP does not significantly change its activating effect on PhK (79). Surprisingly, we found that β -dimer formation was unchanged in the presence of these four known PhK effectors. There are several potential reasons for this, including the possibility that

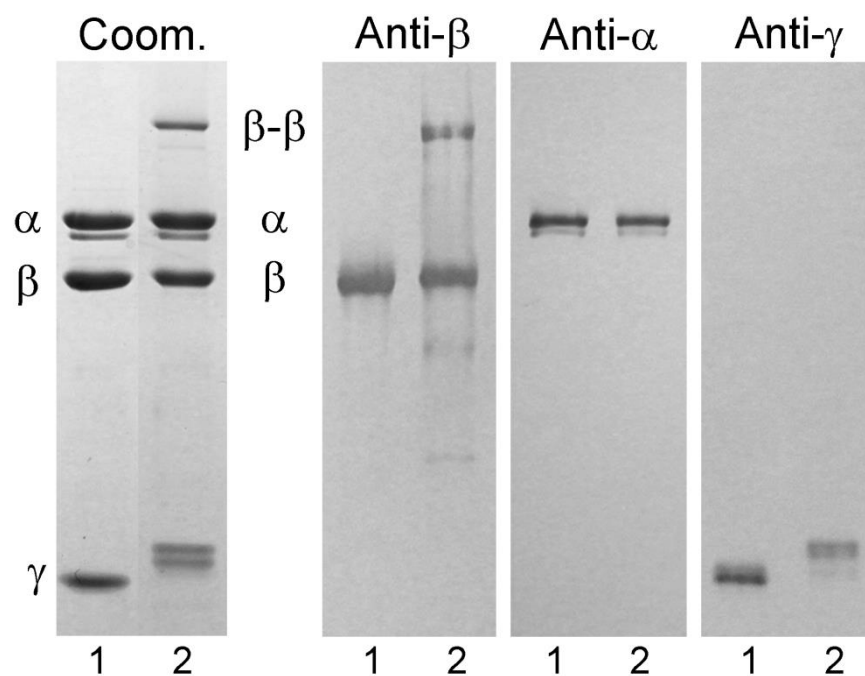


Figure 2.2 Immunodetection of the α , β , and γ subunits in the products of periodate crosslinking of PhK for 10 min. 1, Native PhK; 2, Crosslinked PhK.

the crosslinked region of the two β subunits does not change in the presence of these specific effectors, despite other conformational changes taking place elsewhere in the β subunits, as indicated by past studies (44, 120). Another possibility is that the effector binding sites on the enzyme are being oxidized, altering or eliminating binding before an effect on crosslinking is seen.

N β Peptides and β Crosslinking

The β subunits are the structural and regulatory core of the PhK enzyme complex (47), and their N-termini have been shown to crosslink to the catalytic γ subunit, although the crosslinker used was relatively long (55). The synthetic N β peptide inhibited that β - γ crosslinking and was, in fact, crosslinked to γ itself, *i.e.*, even within the PhK complex it mimics and competes with the N-termini of the β subunits to which it corresponds (55). Importantly, N β inhibits the kinase activity of phospho-activated PhK, but not that of non-activated PhK, while phospho-N β inhibits neither form of the kinase (55). The sum of these results suggests that phosphorylation of the N-terminal region of β diminishes its interaction with γ . Given that phosphorylation of the N-terminus of β also promotes β self-association (46, 122), we hypothesized that the N β peptide, by mimicking and competing with the N-termini of the β subunits, would disrupt β - γ interactions in the non-activated (non-phosphorylated) holoenzyme complex, thus inducing an increase in β -dimer formation by periodate. As predicted, periodate crosslinking of PhK in the presence of the N β peptide caused an increase in β -dimer formation (Figure 2.3). Also as expected, phosphorylated N β peptide was considerably less effective in promoting β -dimer formation (Figure 2.3), which is consistent with our hypothesis that phosphorylation of the peptide inhibits its ability to compete with the native N-terminus of β in interacting with the γ subunit.

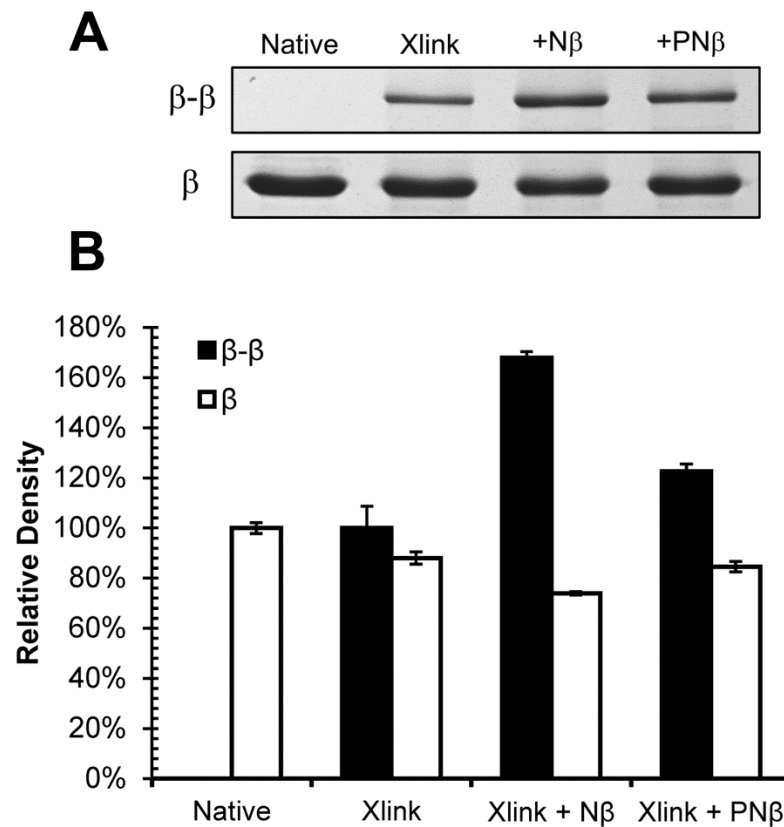


Figure 2.3 N β peptide and periodate crosslinking of the β subunit of PhK. (A) SDS-PAGE of PhK treated with 500 μ M periodate for 2.5 min in the presence of the N β peptide or phosphorylated N β peptide (1 mol $\alpha\beta\gamma\delta$: 100 mol peptide). (B) Density of the β and β - β bands for the different conditions. The β densities are normalized to the monomeric β in native PhK and the β - β densities are normalized to the β - β in control crosslinked PhK (Xlink). Error bars represent standard deviation of quadruplicate samples.

Discussion

Herein we report the selective crosslinking of the large regulatory β subunits of PhK by the general oxidizing agent periodate. Although this oxidant is frequently used to cleave the carbon-carbon bond between vicinal hydroxyl groups in saccharides, we have successfully used low concentrations of periodate ($\geq 10 \mu\text{M}$) to oxidize susceptible amino acid side chains and thus introduce a zero-length crosslink into the hexadecameric structure of non-activated PhK, allowing the study of β subunit interactions. Although it would be useful to know the exact amino acids in β that are crosslinked, which we have achieved with other PhK crosslinkers (55, 129), that would not be trivial with periodate, given that it likely modifies numerous residues not involved in crosslinking. Periodate has been reported to oxidize the side chains of Tyr, Met, Trp, Cys, Asp, Asn, Arg and His (130, 131), but the only crosslink that has been characterized is a 3,3'-dityrosine that it formed in ovotransferrin (132, 133). A variety of crosslinking reagents has been successfully used to study subunit interactions in PhK, but only two of these have been zero-length, forming α - α , α - β and γ - δ dimers (53, 134, 135). Zero-length crosslinkers are desirable because they demonstrate that two subunits directly interact, as opposed to being only proximal. The only crosslinker previously shown to be selective for PhK's β subunits was 1,5-difluoro-2,4-dinitrobenzene (DFDNB), which has a 3-5 Å spacer arm and also forms β - β dimers, but only in small amounts and only with activated conformers of PhK (79, 122). Because the four β subunits form the four central bridges that interconnect PhK's 2 lobes (45, 47), the β -dimers formed by periodate or DFDNB could be either intralobal or interlobal, as the subunits could potentially interact with each other within or across each lobe. Although we do not know the specific regions of β that DFDNB and periodate crosslink to form β -dimers, it is likely that they target distinct areas of the subunit given their different responses to effectors of PhK on their crosslinking (122).

The quaternary structure of non-activated PhK inhibits the activity of its catalytic γ subunit (*i.e.*, quaternary constraint) (100, 136, 137), and subunit interactions within the complex are altered concomitant with enzyme activation (*i.e.*, relief of quaternary constraint) (46, 54, 55, 122). Overall, subunit interactions within the PhK complex are destabilized by phosphorylation, except for those of its β subunits, whose self-association is strengthened (46), and it is the phosphorylation of β that is the key activator of PhK (104, 137). Within the hexadecameric PhK complex, regions of the β and γ subunits have been shown to be not only proximal (55), but to be structurally coupled to each other and with enzyme activation (120). Moreover, in native MS, β - β dimers are observed only in phospho-activated PhK, whereas β - γ dimers are present in only non-activated PhK (46). Thus, findings using multiple techniques link the phosphorylation of the N-terminus of β with activation of γ and increased self-association of β . The initiator of these linked events is the phosphorylation of the N-terminus of β , and this region has, in fact, been shown to control β dimerization. In yeast two-hybrid experiments designed to detect self-association of β to form homo-dimers, no self-association was observed with full-length β ; however, removal of the first 31 residues from its N-terminus or phospho-mimetic Ser→Glu mutations in the N-terminus of full length β both led to homo-dimerization (55). So, how might phosphorylation of this region of β relate to activation of γ ? In addition to its being zero-length, another major utility of periodate's crosslinking of PhK is that it targets a region of β that is sensitive to the interactions of its N-terminus (Figure 2.3), the site at which activating phosphorylation occurs. The sum of the above facts allows construction of a theoretical low resolution structural model for the activation of PhK by phosphorylation.

In this model (Figure 2.4), we envision for the non-activated enzyme a direct interaction between the γ subunit and the N-terminus of β . This β - γ interaction can be thought of as an equilibrium between the non-activated state of PhK (intact β - γ interaction and weak β subunit association) and an activated state (no, or limited, interaction between γ and the N-terminus of β

and enhanced β subunit self-association). In the non-phosphorylated enzyme, the β - γ interaction would stabilize the non-activated state of γ (Figure 2.4A). The above scenario is consistent with the findings that both the N-terminus of β and the N β peptide crosslink to the C-terminal regulatory region of γ , albeit with a 7.3-10 Å crosslinker (55). Previously, from results with peptide mimetics, the N-terminus of β has been hypothesized to inhibit non-activated PhK through either a direct or indirect interaction with γ (55, 138). In our model, successful competition of the N β peptide for the binding site on γ for the N-terminus of the β subunit would free that N-terminus and thus strengthen β self-association (Figures 2.3 and 2.4B); however, the γ subunit would remain in the non-activated state because it would still have the N β peptide bound to it (Figure 2.4B). Consistent with this last point, the N β peptide has no effect on the activity of non-phosphorylated PhK (55). Phosphorylation of the N β peptide is predicted to impair its binding to γ , thus it would compete poorly with the N-terminus of the γ subunit, leaving γ in the non-activated state and not strongly promoting β self-association (Figures 2.3 and 2.4C). Finally, we propose that phosphorylation of the N-terminus of β at Ser11 or Ser26 disrupts the β - γ interaction, again leading to activation of γ and enhanced β self-association (Figure 2.4D). Our model predicts that the N β peptide could readily bind to the γ subunit in this state and bring about inhibition, which is the case (55). It should be noted that in this model enhanced β self-association is a consequence of activation, not its cause. It is attenuation of the interaction between the catalytic γ subunit and the N-terminus of β that gives

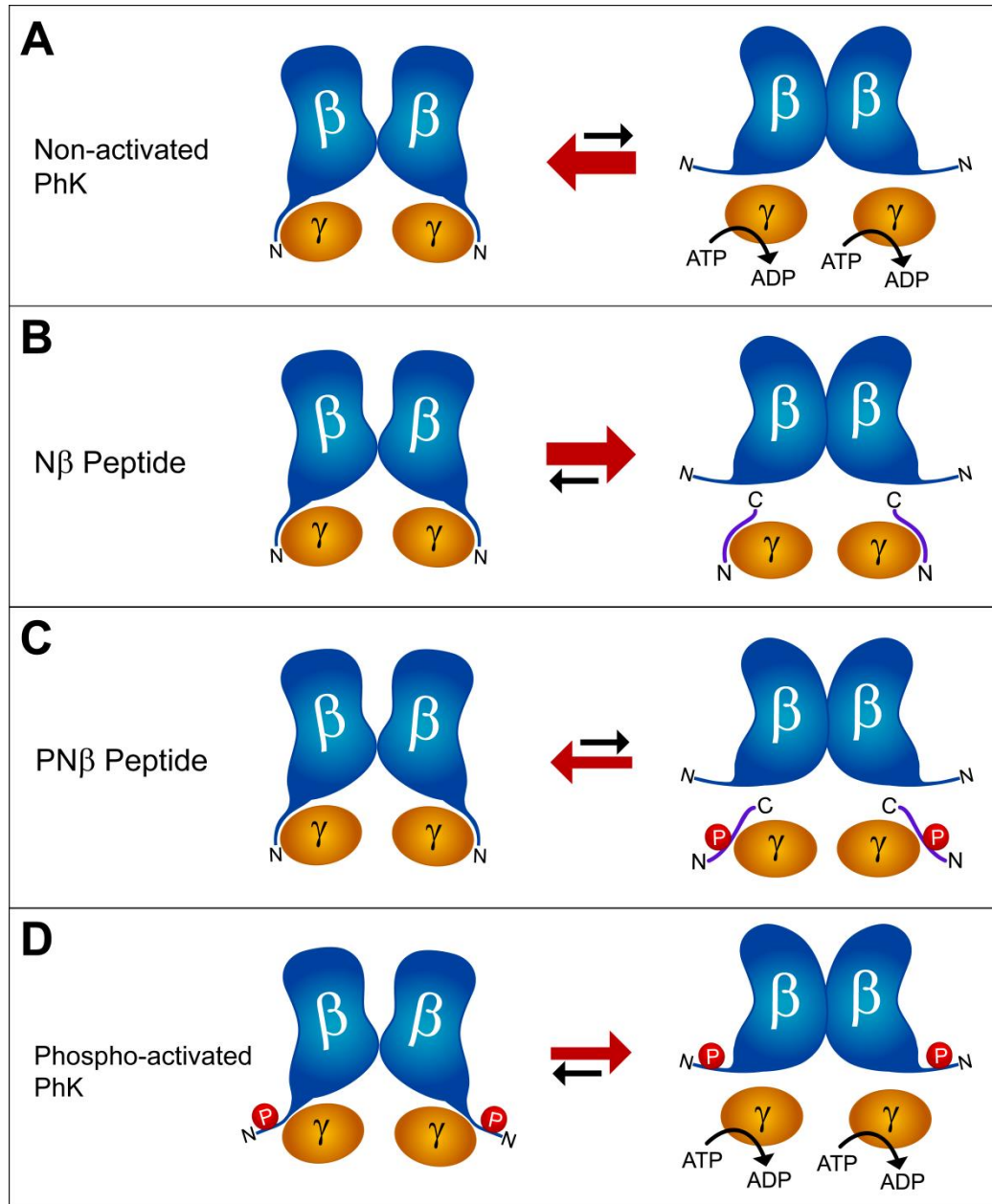


Figure 2.4 Model for Activation of PhK. We propose a model for activation of PhK mediated by the phosphorylatable N-terminus of the large, regulatory β subunits. In the non-activated enzyme complex (A), the unphosphorylated N-terminus of β interacts with the γ subunit, inhibiting kinase activity. In the presence of a competing peptide (N β), or phosphorylation, the N-terminus of β loses strong contact with γ and promotes self-association of the β subunits (B and D). The phosphorylated N β peptide does not compete as well for the interaction with γ (C).

rise to activation and the concomitant β -dimerization detected by periodate oxidation. Thus, β -dimers can be formed independently of activation when the N β peptide competes with and displaces the N-terminus of the β subunit from γ (Figure 2.3).

Materials and Methods

PhK and N β Peptide

Non-activated PhK was purified from the psoas muscle of female New Zealand White rabbits, dialyzed into 50 mM HEPES (pH 6.8), 0.2 mM EDTA, and 10% sucrose (w/v), and stored at -80 °C (75). The concentration of PhK was determined spectrophotometrically at 280 nm using an absorbance coefficient of 1.24 mL mg⁻¹cm⁻¹ (137). The N β and phosphorylated N β peptides were purchased from Biopeptide (San Diego, CA), and their purity and composition verified by both MS and MS/MS analyses using a MALDI 4700 mass spectrometer in the Mass Spectrometry Facility of the University of Kansas Medical Center.

Oxidative Crosslinking

Sodium meta-periodate (99.8+%, CAS No. 7790-28-5) was used as received from Acros Organics. Non-activated PhK was incubated at 30 °C for 2 min before periodate was added to initiate the crosslinking reaction. The standard final reaction mixture contained 20 mM HEPES (pH 6.8), 0.1 mM EDTA, 500 μ M periodate and 1.35 μ M $\alpha\beta\gamma\delta$ PhK protomer. When present, the peptide concentration was 135 μ M. At appropriate times, aliquots of the reactions were removed and quenched in 125 mM Tris, 20% (v/v) glycerol, 5% (v/v) β -mercaptoethanol, 4% (w/v) SDS, and trace Coomassie R250 (pH 6.8). Quenched samples were run on 6-18% gradient polyacrylamide gels, followed by staining with R250 Coomassie (0.1%) and Bismark Brown (0.02%) in 7% acetic acid and 40% methanol. Gels were destained in 7% acetic acid and 5% methanol. Crosslinking reactions were carried out at least in triplicate with multiple preparations

of PhK to confirm the reproducibility of the crosslinking. The density of the PhK subunits and crosslinked species was determined using ImageJ software (139).

To prepare phospho-activated PhK for crosslinking, the phosphorylation reaction (total volume of 80 μ L) contained 54 mM β -glycerophosphate (pH 7.0), 0.5 mM EGTA, 0.01 mM DTT, 50 mM NaF, 1.5 mM Mg(OAc)₂, 0.25 mM [γ -³²P] ATP, 750 μ g/mL PhK, and 2 μ g/mL PKA (murine catalytic subunit, New England Biolabs, Cat. No. P6000L). The reaction was initiated by addition of MgATP, run at 30 °C for 1 min, and quenched with 20 mM EDTA. The phosphate incorporation into β was 4x that into α (0.32 vs. 0.083 mol P/ mol subunit). The quenched, phosphorylated PhK was immediately diluted into buffer containing a final concentration of 510 μ M periodate and 437 μ g/mL PhK. Crosslinking was carried out for 10 min at 30 °C and quenched in SDS buffer. After running the samples on a 6-18% polyacrylamide gel, staining, and destaining, the α , β , and crosslinked product bands were excised, decolorized in 350 μ L 30% H₂O₂ at 104 °C for 1 h, and diluted in 4 mL scintillation fluid. The specific radioactivity of the bands were compared between crosslinked and non-crosslinked phospho-activated PhK.

Apparent Molecular Mass Determination

The apparent molecular masses of the crosslinked products were determined based on the relative mobilities on SDS-PAGE gels of the native PhK subunits and protein standards. Using a broad range, molecular mass marker mix from Bio Rad (cat no. 161-0318), the relative mobility was plotted against the log of the molecular mass for each protein or subunit. The resulting line had an R value of 0.99 and the corresponding linear equation was used to calculate the molecular mass of the crosslinked species from its relative mobility. For the theoretical masses of a β -dimer and a $\beta\gamma\gamma$ -trimer, the apparent molecular masses of each subunit were used, *i.e.* the mass based on the relative mobility and not the actual mass of the subunits from amino acid compositions.

Western Blots

Samples for immunodetection were transferred from the SDS-PAGE gels to PVDF membranes and blocked with 5% (w/v) nonfat, powdered milk, PBS (0.14 M NaCl, 2.7 mM KCl, 6 mM P_i) (pH 7.4), 0.1% Tween20, and 0.2% gelatin. The primary mAbs against the α , β , and γ subunits of PhK have been previously characterized and were used as described (43, 120). Colorimetric detection of the immunoreactive bands was performed with AP-conjugated secondary antibodies from Southern Biotechnology.

N-Terminal Sequencing

Crosslinked PhK was run on SDS-PAGE, blotted onto PVDF, and stained with Ponceau S before the bands were excised and submitted to the Harvard University Mass Spectrometry and Proteomics Resource Laboratory (Cambridge, MA) for N-terminal sequencing using Edman sequencing analysis.

Chapter III: The regulatory α and β subunits of phosphorylase kinase directly interact with its substrate, glycogen phosphorylase

A portion of this chapter has previously been published and is reprinted here with permission. Thompson, J. A., Carlson, G. M. (2017) The regulatory α and β subunits of phosphorylase kinase directly interact with its substrate, glycogen phosphorylase. *Biochem Biophys Res Commun.* 482, 221-5.

Introduction

The breakdown of glycogen is mediated by phosphorylase kinase (PhK) and glycogen phosphorylase (GP). PhK phosphorylates a single serine on GP, causing activation and subsequent phosphorolysis of glycogen to release glucose-1-P (140). PhK is also regulated by reversible phosphorylation, and both GP and PhK are also regulated by a variety of allosteric effectors (rev in (12, 141)). This complex regulation of PhK and GP is consistent with their large masses. PhK is a hexadecameric complex having four copies of four different subunits (α , β , γ and δ) and a total mass of 1.3 MDa (42). The γ subunit (44.7 kDa (49)) is catalytic, and the remaining three subunits, α (138.4 kDa (67)), β (125.2 kDa (63)) and δ (16.7 kDa (142)), are regulatory (100), with the δ subunit being a molecule of non-dissociable calmodulin (42). The substrate GP is a homodimer of 197 kDa (29). The GP dimer has two distinct faces: a convex regulatory face, where the phosphorylatable serine and allosteric binding sites reside, and a concave catalytic face (18).

The recognition of protein substrates by kinases can be complex. The selective phosphorylation of protein substrates depends not only on having appropriate amino acids surrounding the residue to be phosphorylated (primary structure), but also frequently on docking site interactions distinct from the phosphorylation site(s) (112). After decades of extensive work with peptide substrates, consensus amino acid sequences preferred by most kinases are reasonably well-defined, but docking site interactions between kinases and their substrates are by comparison poorly understood (112, 144, 145). These distinct contact points often play important roles in substrate recognition and phosphorylation (144, 145).

Unlike most other kinases, PhK has only one recognized physiological target, GP. Moreover, GP is known to be phosphorylated only by PhK, making this an unusually specific kinase-substrate pair. Despite this unusual specificity and the importance of these two enzymes, little is known regarding the physical interaction between PhK and GP, and their large sizes complicates traditional binding and structural studies. We know that the N-terminus of GP, which contains its single phosphorylation site, must bind to the active site of γ to be phosphorylated, and the crystal structure of truncated γ with a bound peptide substrate reveals details of this binding (51). Importantly, the only comprehensive binding studies on the interactions between PhK and GP found that GP lacking its phosphorylatable N-terminus still bound to PhK with a similar affinity as full-length GP (113), suggesting additional contact site(s). Yeast two-hybrid studies raised the possibility that a C-terminal region the regulatory α subunit (residues 864-1014) of PhK may interact with the N-terminal half of GP (residues 17-484) (114). Calmodulin, PhK's δ subunit, has also been shown to bind an N-terminal fragment of GP (115). Only the β subunit has not been previously implicated in the binding of GP. Here we report the use of short chemical crosslinkers to unambiguously show direct interactions between GP and the two large regulatory subunits of PhK, α and β .

Results and Discussion

Identifying GP-PhK crosslinkers

To limit the amount of crosslinking, a two-step crosslinking approach was used to capture interactions between GP and the subunits of PhK. This approach involved pre-labeling GP with a low concentration of crosslinker (step 1) immediately prior to incubation with PhK (step 2). The pre-labeling of GP preferentially captured conjugates between PhK and GP before intramolecular crosslinking within the GP dimer or PhK hexadecamer could dominate. Of seven different crosslinking reagents screened using this two-step method, four (sulfosuccinimidyl 4,4'-azipentanoate; bis(sulfosuccinimidyl)suberate; N- α -maleimidoacet-oxysuccinimide ester; and

formaldehyde) failed to capture GP-PhK conjugates, but three (1,5-difluoro-2,4-dinitrobenzene (DFDNB), succinimidyl iodoacetate (SIA), and N-5-azido-2-nitrobenzoyloxysuccinimide (ANB-NOS)) successfully did so.

Several major products are formed from DFDNB crosslinking of GP and PhK. The two most intense crosslinked bands are intramolecular PhK products, formed with or without GP present (Figure 3.1A, lane 4). One is a slower migrating α - β conjugate, and the other is an intra-subunit crosslink of β (Figure 3.1B). When DFDNB, PhK and GP are present together, two new bands form below the α - β product (Figure 3.1A, lane 3), the slower being a doublet that cross-reacts with anti-GP and anti- α antibodies, identifying it as a GP- α conjugate. The faster migrating band is a GP- β conjugate. There are additional PhK-GP products above the α - β band; however, these are not well-defined, and their composition is difficult to confidently identify by Western blot due to their proximity to one another. Finally, GP undergoes a small amount of inter-subunit crosslinking to form three, faint bands (Figure 3.1A, lane 2).

Another crosslinking reagent, SIA, also formed GP-PhK conjugates (Figure 3.2A). While GP and PhK were both independently crosslinked by SIA (Figure 3.2A, lanes 1 and 3), when they were present together, three new products formed (lane 2). Western blotting identified the products as the conjugates of GP- α , GP- β , and a second, faster migrating GP- β (GP- β_F) (Figure 3.2A, bottom panel). The GP- α product is a doublet, like the GP- α formed by DFDNB, suggesting that multiple sites are crosslinked between GP and α .

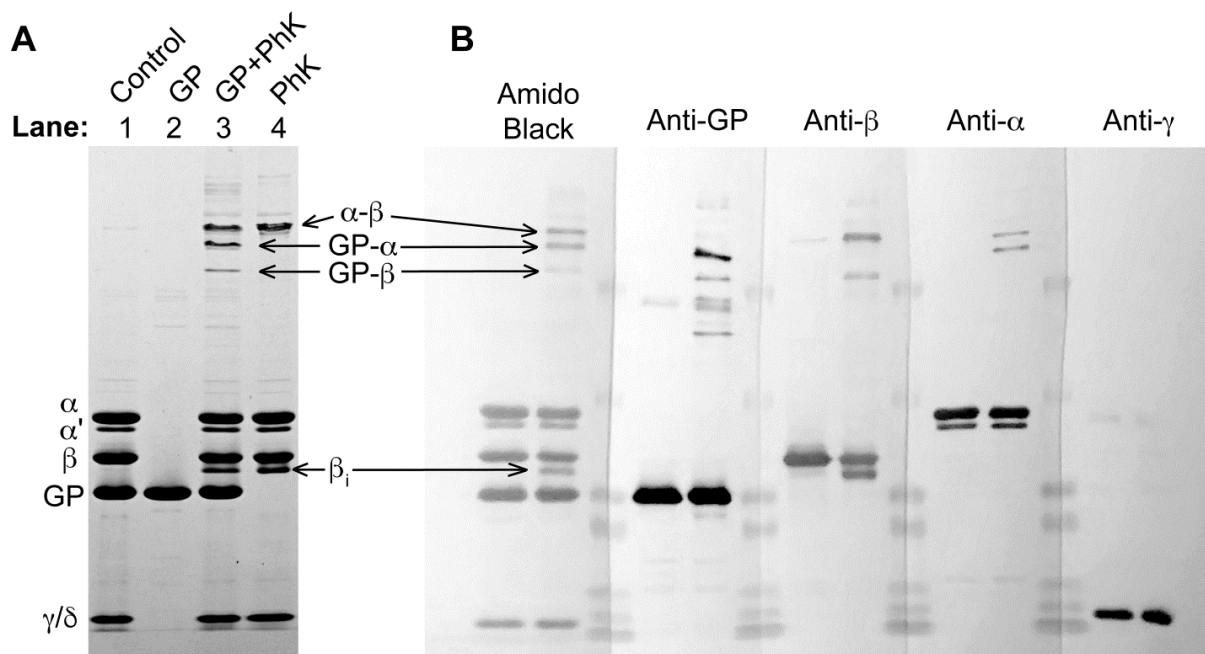


Figure 3.1 DFDNB crosslinking of GP and PhK. (A) Coomassie stained 4-12% SDS-PAGE gel with control and crosslinked PhK \pm GP. Lane 1 is PhK and GP without DFDNB crosslinking. PhK or GP were crosslinked individually to determine intramolecular crosslinking by DFDNB (lanes 2 and 4). Lane 3 has both GP and PhK present with DFDNB. (B) Amido black staining and Western blot of PhK and GP crosslinked together by DFDNB. Previously characterized antibodies to GP and the α , β , and γ subunits of PhK were used to identify the conjugate species (43, 111, 120). β_i , intra-subunit crosslink of β . α' , a naturally occurring splice variant of the α subunit.

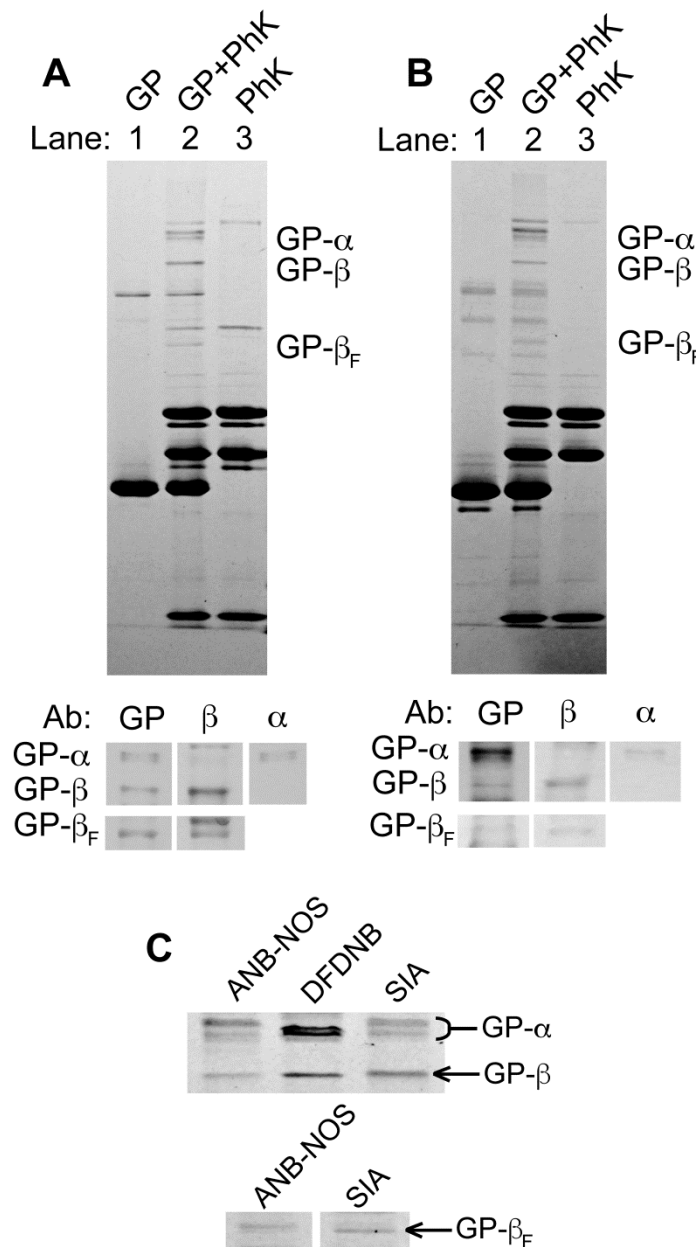


Figure 3.2 SIA and DFDNB crosslinking of GP and PhK. (A) Coomassie-stained 4-12% SDS-PAGE gel and Western blot analysis of SIA crosslinking products. (B), same as (A) but with ANB-NOS crosslinking. (C), Side-by-side comparison of the co-migrating GP- α and GP- β conjugates formed with the different crosslinkers on Coomassie stained 4-12% SDS-PAGE gel.

ANB-NOS, the third crosslinker that formed GP-PhK conjugates, required a different crosslinking approach than used with DFDNB and SIA. ANB-NOS is a photosensitive crosslinker, containing one functional group that remains inert until activated by UV light. Therefore, GP was labeled in the dark by the amine-selective group on ANB-NOS, and unreacted ANB-NOS was removed by gel filtration prior to incubation of the modified GP with PhK. UV light then activated the second functional group on the crosslinker, allowing it to react with bound PhK. Like SIA, ANB-NOS formed three GP-PhK conjugates (Figure 3.2B), which were identified by Western blots as another GP- α doublet, and two GP- β products.

Thus, three different crosslinking reagents apparently form the same GP-PhK conjugates (Figure 3.2C). In the case of the GP- α doublet and the slower migrating GP- β , these conjugates co-migrate for all three crosslinkers. The faster migrating GP- β F appears with SIA and ANB-NOS, but not DFDNB. The consistent formation of these products suggests that the same interfaces between GP and regions of α and β are being sampled by the different crosslinkers and represent genuine contact points between GP and PhK. Moreover, the short spacer lengths of the crosslinkers (1.5 – 7.7 Å) further argues for direct contact of GP with α and β . Our results are consistent with earlier predictions of direct interactions between GP and the regulatory subunits of PhK based on binding studies. GP lacking its phosphorylatable N-terminus binds to PhK in a competitive ELISA assay, but fails to bind the isolated catalytic subunit (113), suggesting that the regulatory subunits of PhK may be involved in the binding of GP. Our results also agree with a previous yeast two-hybrid study that suggested an interaction between GP and PhK's α subunit (114). A direct interaction between GP and the β subunit has not been previously suggested. It is feasible that interactions may also occur between GP and PhK's small, regulatory δ subunit (115), but we were unable to test for the presence of δ in crosslinked conjugates due to the lack of an effective antibody for calmodulin.

Our results directly establish for the first time that there are interactions between GP and regulatory subunits of PhK. The location and function of these newfound interactions may be involved in the unusually selective phosphorylation of GP by PhK, because an isolated form of the catalytic γ subunit lacking its calmodulin-binding C-terminus phosphorylates GP less effectively than does γ within the PhK activated complex. Based on at least five published studies, the average k_{cat} for isolated, truncated γ is 56 sec^{-1} (S.D. ± 26) (69, 73, 117, 146), whereas the average k_{cat} for activated PhK is 245 sec^{-1} (S.D. ± 119) (70, 93, 117, 147-149). Although different laboratories and conditions undoubtedly account for some differences in the k_{cat} values, the reported average value for γ within PhK is nevertheless 4.4-fold greater than that of truncated γ . Thus, despite their inhibition of the γ subunit in non-activated PhK (100), it is possible that the α and β subunits contribute to the catalytic efficiency of γ in the activated complex. The K_m values for GP in the above studies are too scattered to allow a meaningful determination of the specificity constants (k_{cat}/K_m) for the two forms of γ .

Additional binding sites, distinct from the active site, have been identified in other kinase-substrate pairs. Such sites could enhance the interaction between the substrate and active site through several mechanisms (144, 150). For example, regions on GP adjacent in 3D structure to its phosphorylation site could bind to α and β and properly position GP's N-terminus at the active site of γ . Alternatively, GP could also bind α and β at a more distant site, anchoring GP to PhK and increasing the frequency of interaction between GP's N-terminus and the active site. Similar docking or recruitment strategies have been seen with other kinases, including MAPK and cyclin-dependent kinases (151, 152). Another possibility is that GP binding to the regulatory subunits could stabilize a conformation of PhK that promotes phosphorylation through allosteric activation or improved substrate binding, as is seen with PDK1 and MAPK (112, 153).

GP may also bind to α and/or β at a second binding site, completely removed from the active site of PhK. A secondary binding site for GP on the PhK complex, not associated with γ

and phosphorylation, is unprecedented but presents an attractive hypothesis to explain some of the odd and paradoxical features of PhK's interaction with GP. The most striking of these oddities is the 100-fold difference in apparent affinities reported from binding and kinetic studies. The apparent binding affinity for PhK and GP, as measured by a competitive ELISA, was reported to be 29.8 ± 1.3 nM (111) while the K_d derived directly from kinetic rate constants was 5 μ M (70). These two very different apparent affinities may reflect two separate GP binding sites on PhK: a low affinity binding site near the active site and a separate high affinity site on the regulatory subunits. Furthermore, PhK and GP are present in muscle cells at unusually high concentrations, constituting together over 2% of the soluble protein (105, 154). Under these conditions, PhK can phosphorylate all of the GP in just a fraction of a second (155). These high concentrations could potentially drive GP binding to both high and low affinity sites on PhK. The function of a hypothetical second GP binding site is unknown but could be involved in protein folding, stability, or localization of GP and PhK to the glycogen particle. The α and β subunits of PhK may therefore be playing the part of scaffolding proteins, which are used widely by other kinases to localize to their substrates (145, 156). Future work will test the existence of a secondary binding site for GP on PhK and its potential function.

Prior to this, only PhK's catalytic γ subunit was known with certainty to bind GP; however, we did not detect any crosslinking between GP and γ . Active site interactions of protein kinases with their substrates have been suggested to be transient, facilitating rapid turnover (50, 144). Thus, it may be that the interaction between GP and γ is too transient for detection by chemical crosslinking compared to the potentially more stable interactions between GP and the regulatory subunits of PhK.

Effectors and GP-PhK crosslinking

Because *in vitro* phosphorylation of GP by PhK requires Ca^{2+} and MgATP (Mg^{2+} in excess), we determined the effects of these ligands on formation of GP- α and GP- β conjugates during crosslinking with DFDNB. To avoid autophosphorylation, we substituted the nonhydrolyzable analogue adenylyl-imidodiphosphate (AMP-PNP) for ATP. The concentrations of AMP-PNP and Ca^{2+} used were 10-fold above their reported K_d values (58, 157), whereas Mg^{2+} was at a concentration only 6.7-fold above its reported K_a value (158), because of the tendency of PhK to aggregate in the presence of high concentrations of this cation (80). We found that AMP-PNP without divalent cations completely blocked formation of the GP- α and GP- β conjugates (Figure 3.3, compare lanes 4 and 1), whereas inclusion of Mg^{2+} and Ca^{2+} with AMP-PNP partially overcame its protection against formation of the GP-PhK conjugates (compare lanes 4 and 6). Control experiments showed that pre-incubation of AMP-PNP and the cations with DFDNB had no effect on its subsequent reactivity (data not shown), indicating that the effects of these ligands on GP-PhK conjugate formation are due to their direct interactions with at least one of the proteins.

Adenine nucleotide affinity labels modify the α , β and γ subunits of PhK (159-161), and ATP and AMP-PNP bind with similar affinity to γ and an allosteric site (157), most likely on β (159, 160). At higher concentrations, ATP also binds an allosteric site on GP; however, under our conditions, the predicted occupation of the allosteric site by AMP-PNP is sub-saturating (162). Thus, the complete inhibition of GP- α and GP- β formation by AMP-PNP likely results from its binding to the active site or allosteric sites on PhK, inducing conformational changes at the GP

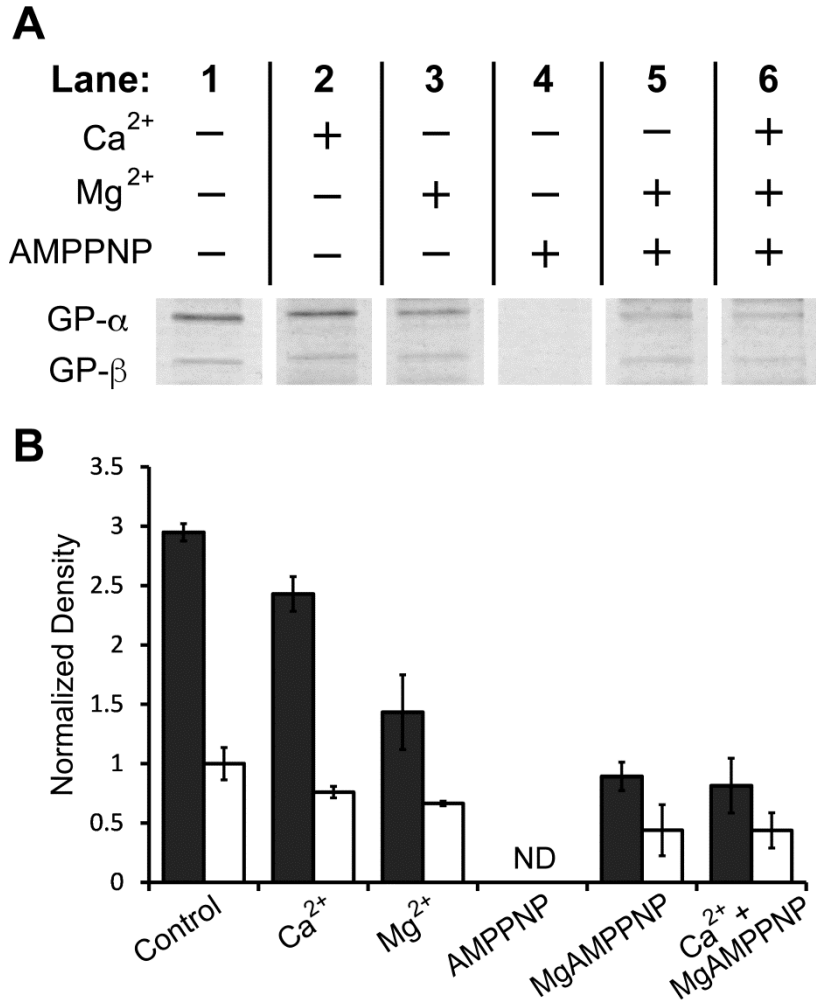


Figure 3.3 DFDNB crosslinking under various conditions. PhK and GP were crosslinked with DFDNB for 5 min in the presence of various effectors after pre-incubation of GP with DFDNB for 2 min. (A) Coomassie-stained GP- α and GP- β conjugates on 4-12% SDS-PAGE gel after crosslinking in the presence of the indicated small molecules. (B) Densities of GP- α and GP- β after crosslinking in the absence or presence of effectors and normalized through dividing by the average density of the control GP- β conjugate. Error bars represent the standard deviation of triplicate samples. The lanes in part (A) came from the same experiment and gel. ND, none detected.

binding sites on α and β . It must be noted, however, that decreased crosslinking does not necessarily indicate loss of interaction. Crosslinking with a short reagent such as DFDNB samples only a small region of the interface between GP and PhK; so, loss of crosslinking could be due to even the smallest structural rearrangements in the interface.

A ligand-induced structural rearrangement is also apparently the reason that both free Ca^{2+} and Mg^{2+} inhibit formation of GP- α and GP- β conjugates by DFDNB (Figure 3.3, compare lanes 2/3 and 1), given that both of these cations actually enhance PhK's binding of GP (111). Free Mg^{2+} stimulates PhK's activity (163) through binding to the γ subunit (164); it also induces several physiochemical changes in the PhK complex (80). Ca^{2+} also stimulates PhK's activity (57, 165), but by binding to its regulatory δ subunit, an integral molecule of calmodulin (42), and Ca^{2+} brings about global conformational changes throughout the PhK complex (44, 166).

Influence of Phosphorylation on the GP-PhK Interaction

PhK and GP are both subject to phosphorylation, which causes conformational changes associated with activation in each enzyme. Because the crosslinking between GP and PhK was sensitive to effectors necessary for phosphorylation (Ca^{2+} , MgATP), and because the regulatory α and β subunits become phosphorylated, phosphorylation of these subunits may influence their GP binding sites. Therefore, DFDNB crosslinking between GP and the regulatory subunits of phosphorylated PhK (phospho-PhK) was compared to crosslinking with non-activated PhK (Figure 3.4). DFDNB crosslinking of the PhK complex alone is known to change considerably between non-activated and phospho-PhK, especially in the β subunits (122). As anticipated, phosphorylation of PhK caused considerable changes to the observed crosslinking pattern, but also led to the disappearance of the GP- α and GP- β conjugates (Figure 3.4). There are several potential explanations for this loss in crosslinking. One possibility is that phosphorylation of PhK causes

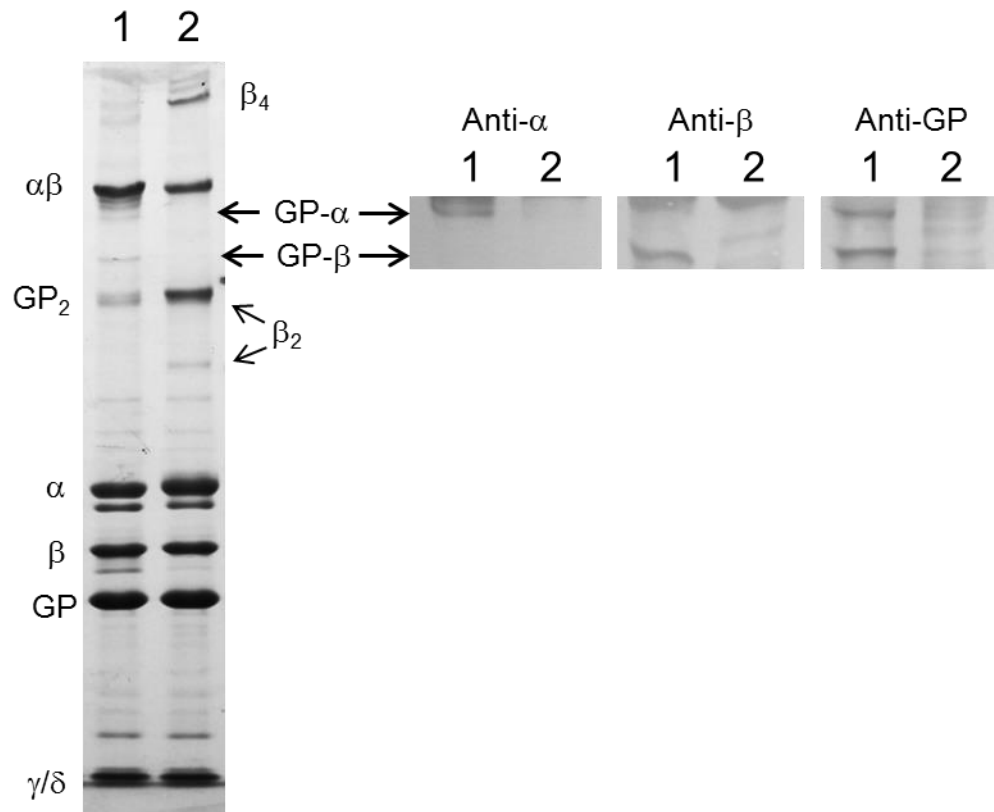


Figure 3.4 DFDNB crosslinking of GP and phospho-PhK. On the left, Coomassie-stained 4-12% SDS-PAGE of DFDNB crosslinking products of GP and non-activated PhK (lane 1) and GP and phospho-PhK (lane 2). PhK was phosphorylated via PKA phosphorylation and crosslinked as described under Materials and Methods. Additional DFDNB crosslinking products have been noted, including the previously observed β dimers and tetramers observed with DFDNB crosslinking of phospho-PhK (124). On the right, Western blot analysis of the GP- α and GP- β products with non-activated and phospho-PhK.

conformational changes at the GP binding sites on α and β that eliminates the crosslinking. Another possibility is that crosslinking of newly exposed sites within phospho-PhK may compete for DFDNB crosslinking at the GP binding sites, leading to a reduction or loss of GP-PhK products. Finally, the migration of the GP- α and GP- β conjugates from phospho-PhK may be different than with non-activated PhK, especially if hypothetical trimers of GP- α - β or GP- β - β are forming instead, which would be difficult to identify due to poor separation of heavy conjugates on the SDS-PAGE (>350kDa). Given the fact that phosphorylated PhK must still interact with GP in order to phosphorylate it, the change seen in DFDNB crosslinking suggests that phosphorylation of PhK may affect how GP is binding to PhK.

GP binding to the regulatory α and β subunits has unknown consequences on the structure and activity of both GP and PhK. Docking of GP on α and β may support its phosphorylation by PhK, but the potential impact of this binding on GP's own conformation is equally interesting and completely unknown. Therefore, GP activity was measured in the absence and presence of non-activated and phospho-PhK (Figure 3.5A). While non-activated PhK appears to slightly inhibit GP's AMP-dependent activity, phospho-PhK appears to have no effect.

The activity of phosphorylated GP was then measured in the presence and absence of non-activated and phospho-PhK (Figure 3.5B). Phosphorylated GP does not require AMP for activity, but is further stimulated by AMP binding. Non-activated PhK again appears to inhibit GP activity (both AMP-dependent and independent). Phospho-PhK, on the other hand, has surprising effects on GP activity. In the absence of AMP, phospho-PhK appears to stimulate GP's activity; but in the presence of AMP, it inhibits activity to the point that activation of phosphorylated GP by AMP is nearly abolished. These results suggest that phosphorylation of PhK and GP may influence how they interact with one another, consistent with the results from GP crosslinking with phospho-PhK.

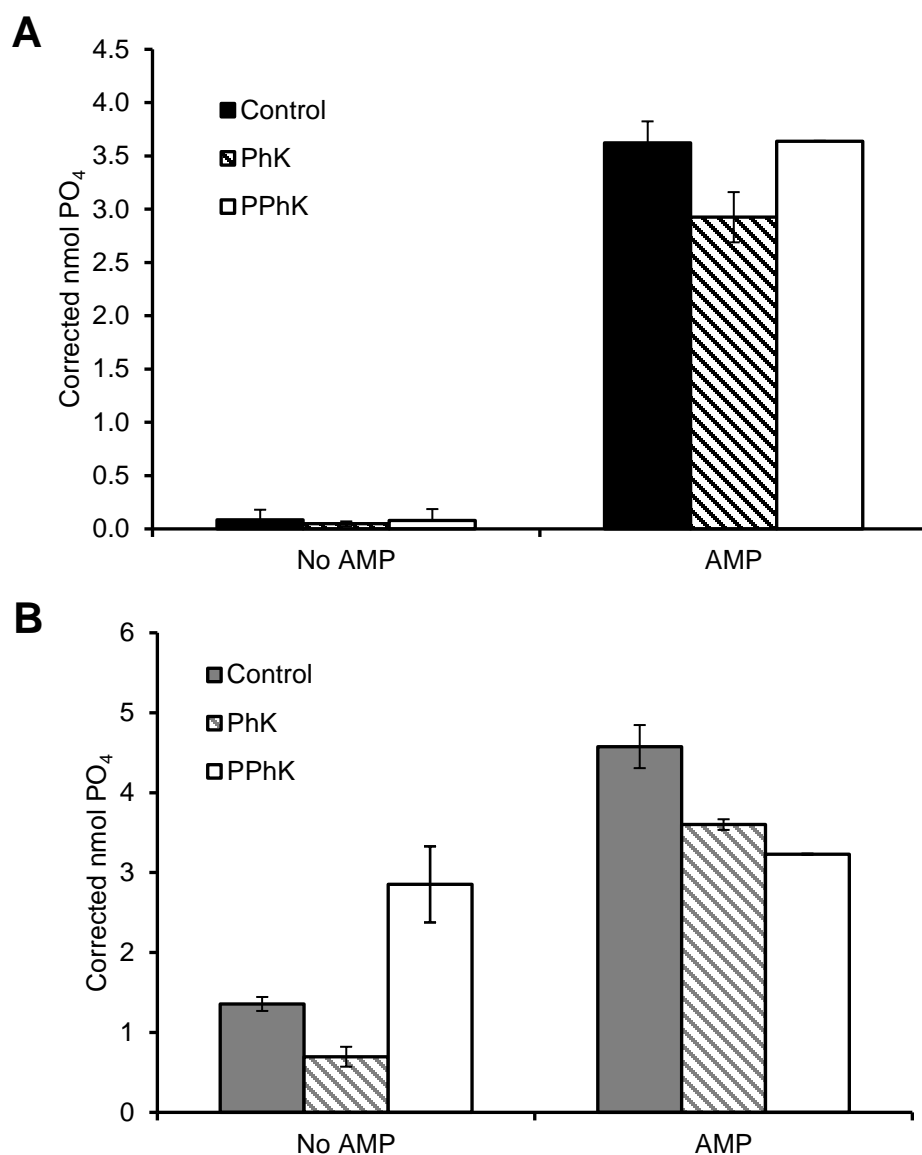


Figure 3.5 GP activity in the absence and presence of non-activated PhK and phospho-PhK (PPhK). (A) GP activity in the absence (black bars) and presence of non-activated PhK (black diagonals) or phospho-PhK (white bars). (B) Phosphorylated GP activity in the absence (gray bars) and presence of non-activated PhK (gray diagonals) or phospho-PhK (white bars). Control GP refers to GP assayed in the absence of PhK. Error bars represent the standard deviation of three reactions. The GP assay was performed as described under Materials and Methods.

Much will be necessary to uncover the structural details and functional significance of these binding sites.

The results of this work suggest that multiple conformational changes are likely associated with the interactions between PhK and GP. That these interactions directly involve regulatory subunits of PhK adds an additional layer of complexity to this already complex, highly regulated system. A deeper understanding of the interaction between PhK and GP and its regulation will help us better understand the strict cellular control of glycogenolysis.

Materials and Methods

Enzymes

Non-activated PhK was purified from New Zealand White rabbit psoas muscle as previously described (75), dialyzed into 50 mM HEPES (pH 6.8), 0.2 mM EDTA, and 10% sucrose (w/v), and stored at -80 °C. PhK concentration was determined spectrally at 280 nm using an absorbance coefficient of 1.24 mL mg⁻¹cm⁻¹ (137). GP was also isolated from New Zealand White rabbit muscle as described previously (34), and recrystallized with Mg²⁺ and AMP. After removal of AMP by dialysis into 10 mM HEPES (pH 6.8), GP was stored at -80 °C. The concentration of GP was determined spectrally at 280 nm with an absorbance coefficient of 1.30 mL mg⁻¹cm⁻¹ (167).

Phospho-PhK was generated by autophosphorylation and PKA phosphorylation for the GP activity and crosslinking experiments, respectively. For PKA-phosphorylation, PhK was phosphorylated for 5 min at 30 °C. Phosphorylation was initiated by the addition of MgATP and the final concentrations were 20 mM Tris/βGP (pH6.8), 0.1 mM EDTA, 0.1 mM DTT, 5 μg/mL PKA, 1 mg/mL PhK, 5 mM Mg(OAc)₂, and 1 mM ATP. The amount of phosphate incorporated was approximately 2 mol PO₄/mol αβγδ. The reaction was quenched with EDTA to a final concentration of 10mM and set on ice before being used directly in a two-step crosslinking with GP. Control PhK was treated in an identical manner except for the absence of ATP. For PhK

autophosphorylation, the reaction was initiated with addition of MgATP and the reaction contained 50 mM HEPES (pH 8.2), 0.05 mM EGTA, 0.1 mM CaCl_2 , 1 mg/mL PhK, 4 mM $\text{Mg}(\text{OAc})_2$, and 0.2 mM ATP. Autophosphorylation for 15 min at 30 °C led to the incorporation of 4 mol PO_4 /mol $\alpha\beta\gamma\delta$. The reaction was quenched with EDTA to a final concentration of 10mM and kept on ice before use in the GP activity assay. Control PhK was treated in an identical manner except for the absence of ATP.

Phosphorylated GP was formed by phosphorylation by PhK. The reaction was initiated by addition of PhK and contained 20 mM Tris/ β GP (pH 8.2), 0.1 mM EGTA, 0.2 mM CaCl_2 , 10 mM $\text{Mg}(\text{OAc})_2$, 1.5 mM ATP, and 1 $\mu\text{g/mL}$ PhK. The reaction was run for 30 min at 30 °C at which point the amount of phosphate incorporated reached a plateau of 0.7 mol PO_4 /mol GP. The reaction was quenched with EDTA to a final concentration of 10mM and kept on ice before use in the GP activity assay. Control GP was treated in the same way, but in the absence of ATP.

Chemical crosslinking

The crosslinkers DFDNB (CAS No. 327-92-4), SIA (CAS No. 39028-27-8), and ANB-NOS (CAS No. 60117-35-3) were from Pierce/ThermoFisher Scientific.

To limit crosslinking, a two-step protocol was used with DFDNB and SIA in the crosslinking of GP with PhK. In step one, GP was pre-incubated with crosslinker (135 μM DFDNB or 540 μM SIA) for 2-5 min at room temperature (RT). The pre-incubated GP was immediately diluted 10-fold into a solution containing PhK and incubated at 30 °C for 5 min. The final concentrations were: 20 mM HEPES (pH 6.8), 0.1 mM EDTA, 437 $\mu\text{g/mL}$ PhK, 260 $\mu\text{g/mL}$ GP, and 13.5 μM DFDNB or 54 μM SIA (carryover solvent was 0.2% DMSO or ACN). The molar ratio of PhK protomer ($\alpha\beta\gamma\delta$) to GP monomer was 1:2, while the ratio of DFDNB and SIA to GP monomer was 5:1 and 20:1, respectively. Reaction aliquots were quenched in an equal volume

of SDS buffer (125mM Tris (pH 6.8), 20% glycerol, 5% β -mercaptoethanol, 4% SDS, and trace Coomassie R250) and analyzed on a 4-12% linear SDS-PAGE gel.

ANB-NOS, a photo-activated crosslinker, was treated differently than DFDNB and SIA. GP was incubated with 100 μ M ANB-NOS for 5 min at RT in the dark. After removal of unreacted ANB-NOS on a P10 de-salting column, the labeled GP was incubated at RT with PhK under a long-wave UV lamp (366nm) at a distance of 1 cm for 5 min. The crosslinking reaction was quenched and analyzed as above.

When effectors were included in the DFDNB crosslinking reaction, they were added to the GP/DFDNB solution 15 sec before addition of PhK. The final concentrations of Mg^{2+} , Ca^{2+} , and AMP-PNP in the second step were 4 mM, 330 μ M, and 300 μ M, respectively. The density of the crosslinked products was determined using ImageJ software (139). To ensure reproducibility, three different preparations of PhK were used, and all experiments were performed in triplicate.

Western blotting

Samples for immunodetection were transferred from SDS-PAGE gels to PVDF membranes and blocked with 5% (w/v) nonfat powdered milk in 0.14 M NaCl, 2.7 mM KCl, 6 mM PO_4 (pH 7.4), 0.1% Tween20, and 0.2% gelatin. The primary antibodies against GP and the α , β , and γ subunits of PhK have been previously characterized and were used as described (43, 111, 120). Colorimetric detection of the immunoreactive bands was performed with AP-conjugated secondary antibodies from Southern Biotechnology.

GP Activity

GP was assayed in the direction of glycogen synthesis by quantifying phosphate cleaved from glucose-1-P. The assay was initiated with addition of GP and the final reaction solution contained 20 mM MOPS (pH 6.8), 2.5 mM glucose-1-P, 0.1 mg/mL glycogen, 3 mg/mL BSA,

and 10 µg/mL GP. When present, the concentration of non-activated or phosphorylated PhK was 500 µg/mL. When present, the concentration of AMP was 1 mM. The glycogen, purchased from Sigma, was further purified by ethanol precipitation and dialysis to remove contaminating nucleotides and phosphate. GP was assayed at 30 °C for 10 min and 2 min for non-phosphorylated and phosphorylated GP, respectively. To quench the reaction, 20 µL aliquots were mixed with 1 mL of freshly prepared malachite green reagent (0.12 mM malachite green, 2.25 mM ammonium molybdate, 830 mM H₂SO₄, 0.02% PVA). The malachite green reaction was allowed to develop for 10 or 12 min and the absorbance at A₆₃₀ was measured. The color development was continuous, so the A₆₃₀ measurements were carefully timed. A standard phosphate curve was generated for each experiment and was linear between 0 and 20 nmol phosphate. Reactions were run in triplicate.

Chapter IV: Activation of phosphorylase kinase by physiological temperature

Reprinted with permission from Herrera, J. E., Thompson, J. A., Rimmer, M. A., Nadeau, O. W., Carlson, G. M. (2015) Activation of Phosphorylase Kinase by Physiological Temperature. *Biochemistry*. 54, 7524-30. Copyright 2015 American Chemical Society. The sections contributed by J.A. Thompson are presented in this chapter in full alongside new, never-before published results.

Introduction

Phosphorylase kinase (PhK) is a large, regulatory enzyme in the glycogenolysis cascade. Decades of research on the PhK hexadecamer ($\alpha\beta\gamma\delta$)₄ *in vitro* have revealed much about this enzyme's structure, activity, and regulation. Fischer and Krebs established the foundation of PhK research in the 1950's, and since then, virtually every PhK study has utilized the same isoform isolated from the fast-twitch muscle of female New Zealand White rabbits. Based on cDNA sequencing, at least four other isoforms of PhK are present in other tissues, such as slow-twitch muscle, liver, and brain, that we know very little about (168). In addition to the widespread focus on the muscle isoform of PhK, the vast majority of experiments performed on the enzyme have been conducted at 30 °C. However, the body temperature of a rabbit is nearly 10 °C higher at 39.7 °C.

Recently, the first report investigating PhK's kinase activity as a function of temperature revealed unexpected activation of PhK at 40 °C (81). PhK's activity increased ~3.5 fold every 10 °C between 0 and 30 °C but from 30 to 40 °C, an 8-fold increase in activity was observed (Figure 4.1A and B). This deviation from the expected increase in reaction rate is clearly evident as a break in the otherwise linear Arrhenius plot (Figure 4.1C). This activation may be caused by a temperature-dependent conformational change in the enzyme, consistent with this temperature-dependent effect being reversible (Figure 4.1E). The implications of this temperature-induced changes in PhK's structure and activity are just beginning to unfold. Thus

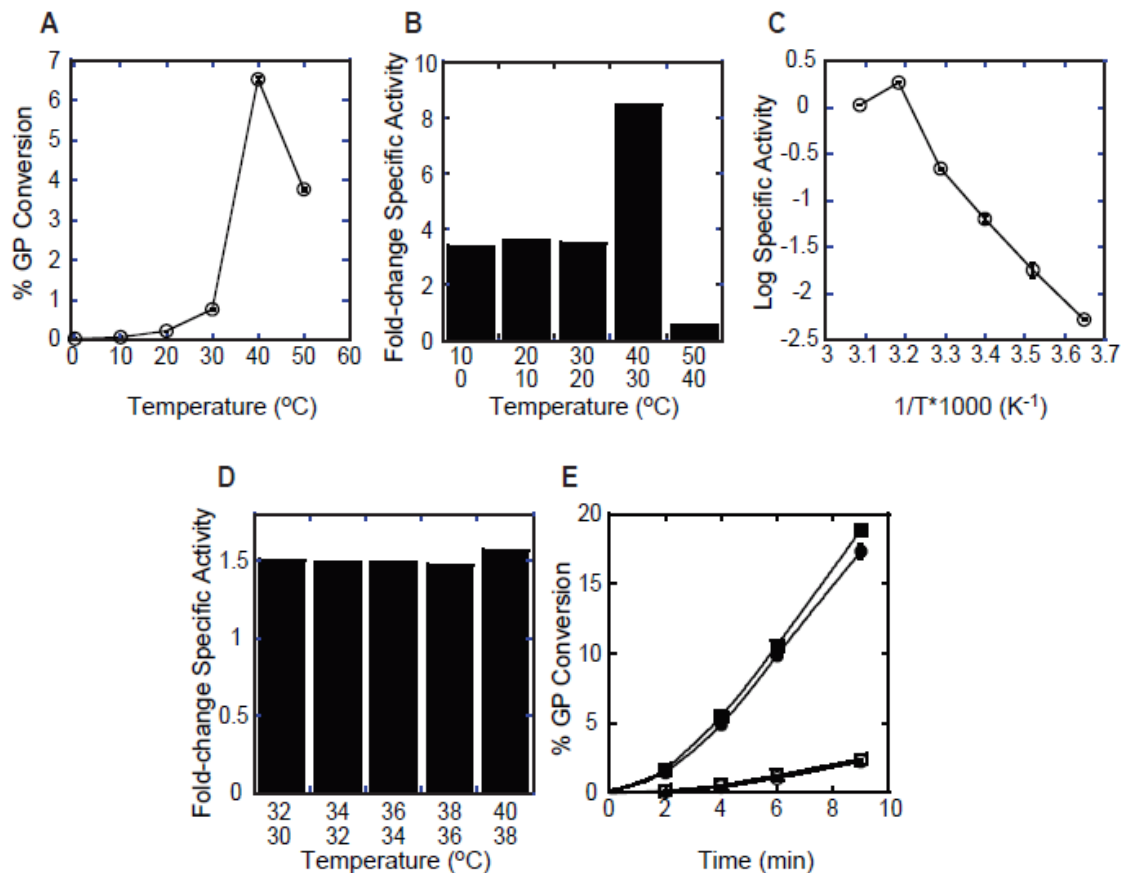


Figure 4.1 Temperature dependence of GP conversion at pH 6.8. (A) The amount of GP converted at a fixed time at each indicated temperature. (B) The fold-change in activity between each 10 °C temperature interval (Q10), calculated by dividing the value at the higher temperature by that at the lower. (C) Arrhenius plot for the activity shown in Panel A. (D) The fold-change in activity at 2 °C temperature intervals (Q2) between 30 and 40 °C, calculated as in Panel B. (E) The effects of pre-incubation and assay temperatures on activity. PhK was pre-incubated for 5 min at either 30 or 40 °C and immediately assayed at both of the same temperatures. The pre-incubation and assay temperatures were 30 and 30 °C (open square), 40 and 30 °C (open circle), 30 and 40 °C (closed square) and 40 and 40 °C (closed circle). Where present, the error bars (red) indicate the standard error of triplicate assays and generally did not exceed the symbol dimensions.

far, activators and inhibitors of PhK appear to have diminished effects on the enzyme at 40 °C vs. 30 °C (81).

One prominent feature of PhK at 40 °C is the near absence of a lag in the product formation (*i.e.*, phosphorylation of GP) (Figure 4.1E). Non-activated PhK displays hysteretic behavior (140), likely related either to a slow conformational change that accompanies activation of the enzyme or to autophosphorylation during the assay. Binding of Ca^{2+} plus Mg^{2+} and autophosphorylation have both been attributed to causing the hysteretic behavior of PhK (75, 90). Activated PhK, assayed at pH 8.2 or phosphorylated by the cAMP-dependent protein kinase (PKA), has a linear rate of product formation.

The near absence of a lag in the PhK reaction at 40 °C creates new opportunities to study the kinetics of PhK, especially given the fact that no information currently exists regarding PhK kinetics at the physiological temperature of 40 °C, and very little is known about the kinetics of non-activated PhK under any conditions, because of its non-linear reaction rate. A previous study by Newsholme and Walsh (73) reported kinetic values for non-activated PhK at pH 6.8, which is considered the physiological pH for rabbit muscle. The authors claimed to have eliminated the lag in activity for non-activated PhK by assaying the enzyme in HEPES buffer, as opposed to Tris/ β GP which is typically used to buffer PhK. When their conditions were repeated at 40 °C, however, a lag was clearly present (data not shown). This suggests that instead of eliminating the lag, their conditions actually lengthened the lag to the point that the initial velocity appeared linear during the timecourse measured.

Sixty years' worth of PhK research can and likely should be re-evaluated in light of the enzyme's surprising activation at 40 °C. To begin, evidence for a temperature-dependent conformational change in PhK will be presented followed by kinetic analysis of both the non-phosphorylated and phosphorylated forms of PhK at 40 °C.

Results and Discussion

Conformational change in PhK between 30 and 40 °C

The surprising rise in PhK activity between 30 and 40 °C may result from a conformational change in the enzyme leading to a more active form at the higher temperature. However, no evidence of a physical change between 30 and 40 °C was obtained from a variety of biophysical techniques including UV absorption spectroscopy, intrinsic Trp fluorescence, far-UV CD, dynamic light scattering, and differential scanning calorimetry (169). Although no structural changes were detected with these techniques, a conformational change is still possible for two reasons: these techniques may have missed small, local conformational changes in the complex and the strong background signals produced by the massive PhK molecule could obscure subtle conformational changes. Attempts to detect a conformational change in PhK between 30 and 40 °C therefore shifted to looking for changes in specific regions of the hexadecameric complex. PKA phosphorylation of α and β was therefore measured at 30 and 40 °C to see if PKA distinguished between PhK conformers at the two temperatures (Figure 4.2). For the α subunit, there was an expected increase in the rate of phosphorylation by PKA (275% increase) at 40 °C that one would typically expect due to an increase in temperature from 30 to 40 °C. In contrast, the β subunit saw only a modest increase in phosphorylation by PKA (30% increase) at 40 °C compared to 30 °C. This difference in the rates of phosphorylation suggests a conformational change at the phosphorylation sites of β between 30 and 40 °C with the higher temperature conformer being a worse substrate for PKA, offsetting the expected increase in phosphorylation one would expect from increasing the temperature.

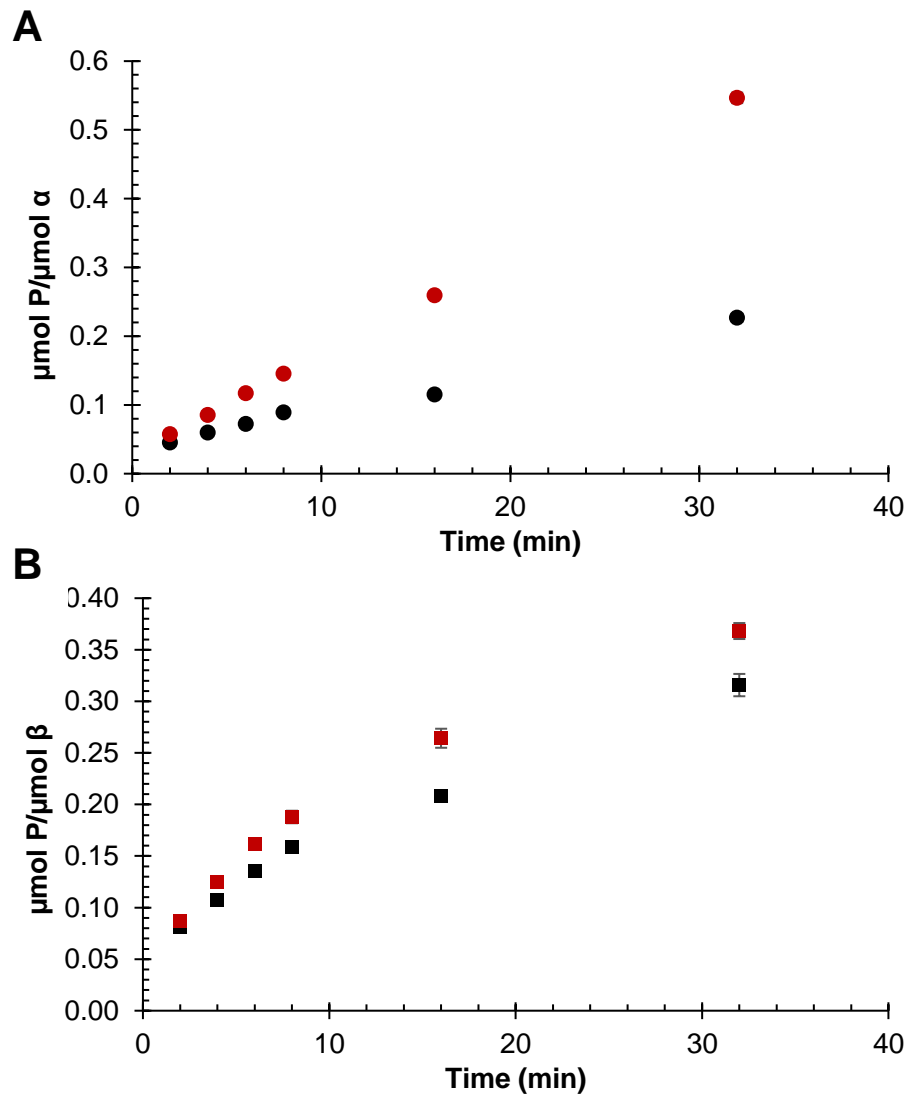


Figure 4.2 Phosphorylation of the α and β subunits by PKA at 30 and 40 °C. (A) PKA phosphorylation of the α subunit at 30 °C (black circles) and 40 °C (red circles). (B) PKA phosphorylation of the β subunit at 30 °C (black squares) and 40 °C (red squares). The reaction was performed as described under Materials and Methods. Assays were performed in triplicate. Error bars depicting the standard deviation generally did not exceed the symbol dimensions.

To further investigate conformational changes in PhK due to increasing temperature, we next turned to chemical crosslinking as a structural probe. Chemical crosslinking has been used successfully in the past to detect conformational changes in PhK (53, 54, 135). The short, homobifunctional crosslinker 1,5-difluoro-2,4-dinitrobenzene (DFDNB) forms crosslinks between three of the four subunits of PhK (α , β , and γ). PhK was therefore incubated with a 50-fold molar excess of DFDNB for a fixed amount of time at 10 °C intervals between 0 and 40 °C (Figure 4.3). The consumption of the subunits by crosslinking was measured by densitometry. The α subunits showed a steady, unremarkable increase in crosslinking with increasing temperature (Figure 4.4). The crosslinking of the β and γ subunits, however saw little consumption by crosslinking between 0 and 30 °C but between 30 and 40 °C, a dramatic increase in consumption of both β and γ occurred (Figure 4.4). This suggests that between 30 and 40 °C, the β and γ subunits undergo a conformational change likely exposing new crosslinking sites leading to their hastened modification by DFDNB. It should be noted that the β subunit underwent intrasubunit crosslinking by DFDNB, leading to a product running slightly faster on an SDS-PAGE gel (Figure 4.3). This intramolecular crosslinked product was included in the densitometry measurement for monomeric β . As seen in Figure 4.3B, both monomeric β and the intramolecular product were consumed at a greatly increased rate at 40 °C.

The β subunits form the structural core of the PhK hexadecamer, which is arranged as two octamers oriented in a head-to-head conformation, with the β subunits forming four interconnecting bridges between the octamers (47). Several effectors of PhK alter the conformation of the β subunits, including phosphorylation (Chapter II), Ca^{2+} (44, 170), Mg^{2+} (80), and ADP (120).

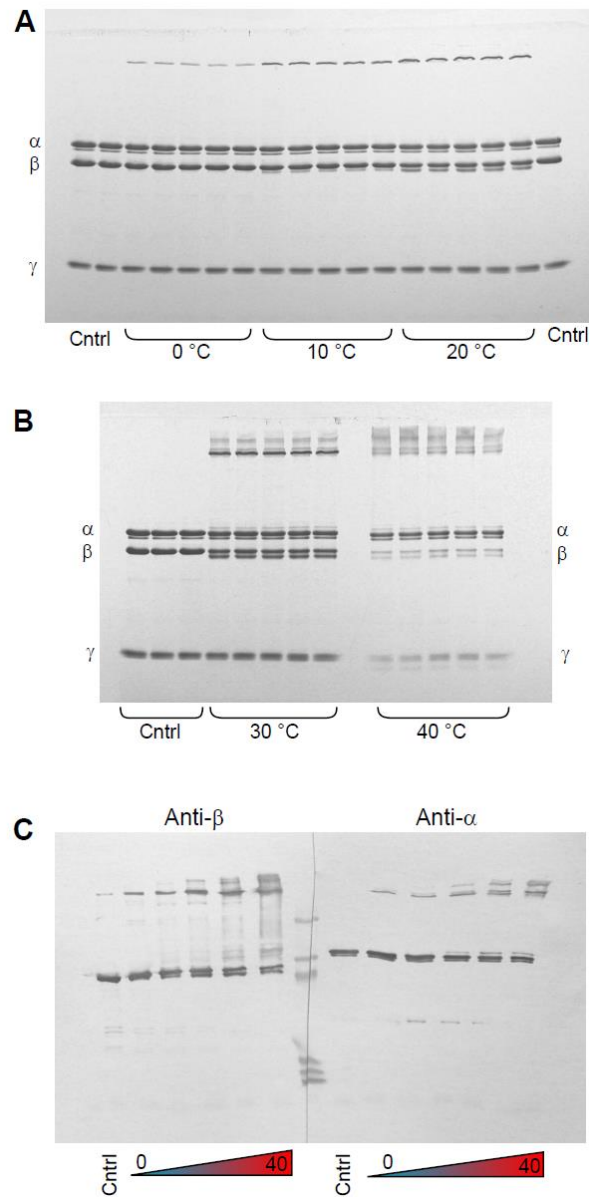


Figure 4.3 Temperature-dependent crosslinking of PhK by DFDNB. PhK was crosslinked by DFDNB as described under Materials and Methods. (A) Non-crosslinked PhK (Cntrl) and PhK crosslinked at 0, 10, and 20 °C, and (B) 30 and 40 °C. (C) Immunodetection of the β and α subunits in control vs. crosslinked PhK at 0, 10, 20, 30 and 40 °C. Samples from each crosslinking temperature used in generating Panels A and B were run on 6-18% gradient gels, transferred to PVDF membranes and blocked in 5% milk, 1x PBS, 0.1% Tween20, and 0.2% gelatin (pH 7.4). The primary mAbs against the α and β subunits of PhK have been previously characterized and were used as described (2, 3). Colorimetric detection of the immunoreactive bands was performed with AP-conjugated secondary antibodies purchased from Southern Biotechnology.

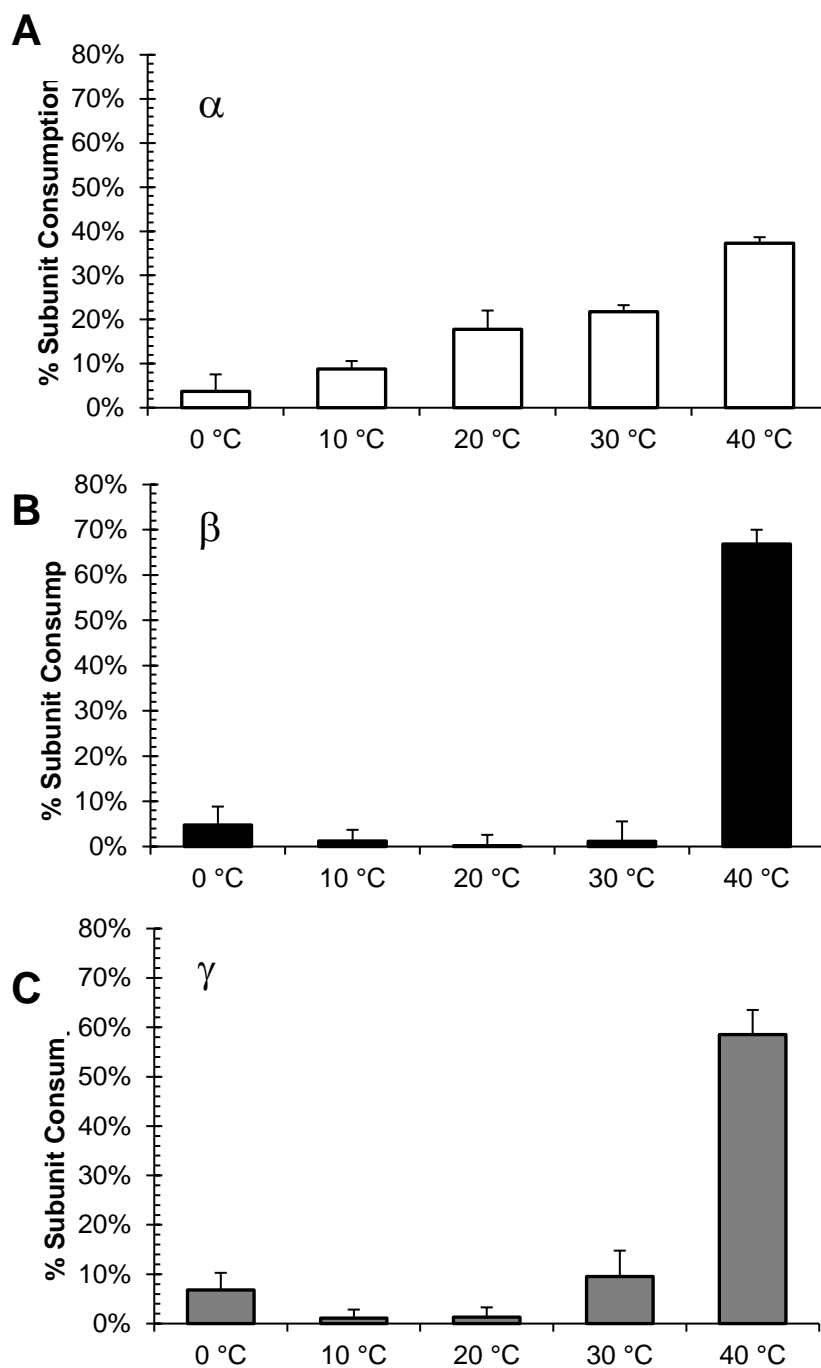


Figure 4.4 Temperature-dependent subunit consumption by DFDNB cross-linking. The percent consumption of α (A), β (B) and γ (C) after 6 min of crosslinking by a 50-fold molar excess of DFDNB at the temperatures indicated. Percent consumption, derived from Figure 4.3, is based on the density remaining of the monomeric subunits relative to the density of the unmodified subunits from the same concentration of control non-crosslinked enzyme. Error bars represent the standard error of 5 replicate crosslinking reactions at each temperature.

Our results show that activation by temperature appears to be another mechanism for activating PhK through the targeting of β .

The β and γ subunits of PhK have been shown to be structurally and functionally coupled to one another. Immuno-electron microscopy revealed that the epitopes of the β and γ subunits are adjacent to one another on the interior lobe face of the complex, and activation of the complex led to parallel increases in antibody binding to β and γ (120). Direct interactions between these two subunits have been supported by multiple techniques including chemical crosslinking and top-down mass spectrometry (46, 55). The proximity of β to the catalytic γ subunit and the reoccurring observation of β conformational changes upon activation by different effectors reiterate the critical role of β in the regulation and activation of PhK.

DFDNB crosslinking was previously shown to crosslink the regulatory α and β subunits of PhK with its substrate glycogen phosphorylase (GP). Given the observation that PhK is likely undergoing a conformational change between 30 and 40 °C and DFDNB crosslinking was sensitive to this change, it seemed worthwhile to determine if the conformational change at 40 °C altered the PhK-GP crosslinking. Using the two-step crosslinking methods described in Chapter III Materials and Methods, PhK and GP were crosslinked for a fixed amount of time at varying temperatures. The first step in crosslinking (treatment of GP with DFDNB) was performed at a constant temperature and the second step (10-fold dilution of GP into the PhK solution) was performed at 10 °C intervals between 0 and 40 °C. Although crosslinking did increase predictably with increasing temperature, no apparent differences in the crosslinking pattern were detected at 40 °C (*i.e.*, GP- α and GP- β conjugate formation remained unchanged) (data not shown).

Kinetics of PhK at Physiological Temperature

Non-activated PhK has eluded kinetic assessment because of its hysteretic behavior. Although PhK's activity and structure differ between 30 and 40 °C, the near absence of

hysteresis allows for the determination of kinetic constants at the physiological temperature and a comparison of changes in $K_m(\text{GP})$ and V_{\max} due to phosphorylation.

Although PhK's lag was greatly reduced at 40 °C, effort was taken to further minimize it. The pH of the assay was therefore raised to 7.2, the top of the physiological range of pH values observed in muscle tissue (171). K^+ was also included in the assay at physiological concentrations and was found to stimulate PhK activity. Previously, anions and to a lesser extent cations, of neutral salts were shown to generally inhibit PhK activity (78). The exception, however, was $(\text{NH}_4)(\text{CH}_3\text{CO}_2)$ which stimulated PhK (78), similar to the stimulation seen in the present studies with $\text{K}(\text{CH}_3\text{CO}_2)$. With these updated conditions, the activity of PhK was nearly linear, especially when compared to the enzyme assayed at 30 °C under the same conditions (Figure 4.5).

Using these conditions, substrate saturation curves were obtained with non-activated PhK at 40 °C and phospho-activated PhK at 30 and 40 °C (Figure 4.6). The data were fit using non-linear least squares fitting in KaleidaGraph, and the kinetic constants are summarized in Table 4.1. Phosphorylation increased the specific activity of PhK at both 30 and 40 °C, but the effect was less pronounced at 40 °C. Only modest changes to $K_m(\text{GP})$ occurred among the conditions tested, implying that activation of PhK by phosphorylation was not accompanied by a drastic change in K_m . This again challenges the early report by Krebs (74) that suggested activation of PhK caused a change in K_m for GP, but agrees with a more recent report by Newsholme (73) who reported a V_{\max} change between non-activated and phospho-activated PhK. The change in V_{\max} between non-activated and phospho-activated PhK in the Newsholme report was much larger than the changes

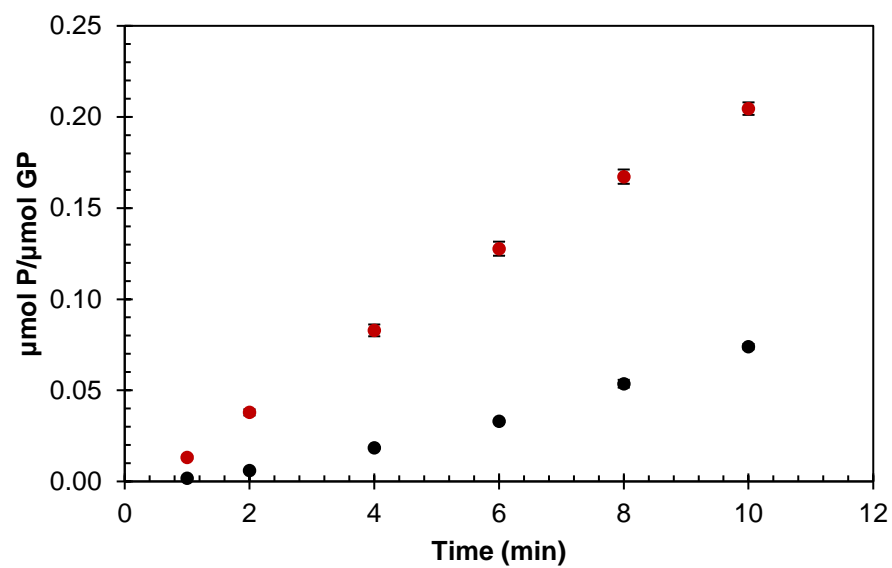


Figure 4.5 PhK activity at 30 and 40 °C. Non-activated PhK was assayed as described under Materials and Methods for a 10-min timecourse at 30 °C (black circles) and 40 °C (red circles) at pH 7.2. Error bars represent the standard deviation of triplicate samples.

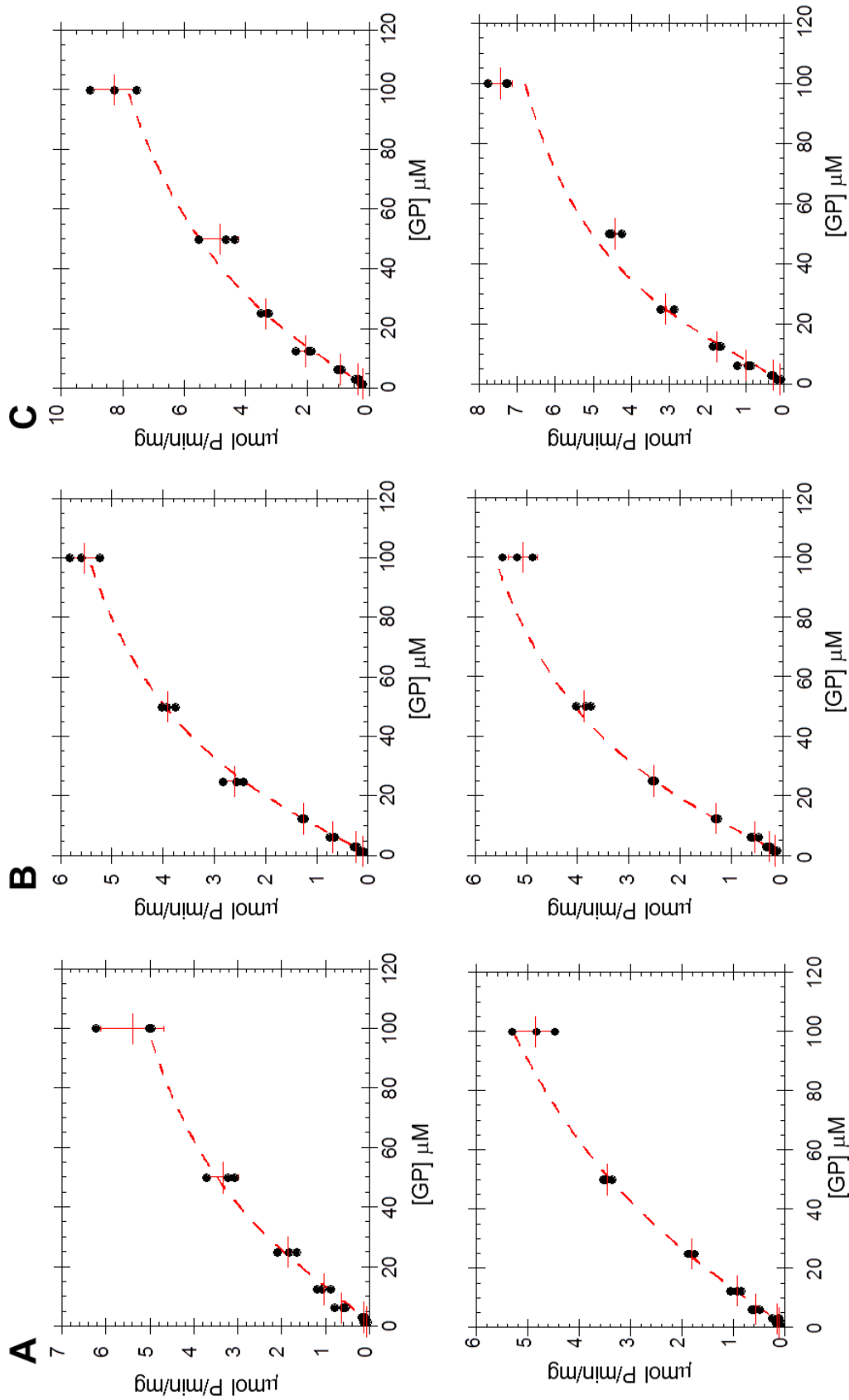


Figure 4.6 GP saturation curves and data fitting. (A) Specific activity at various concentrations of GP for phosphorylated PhK at 30 °C. The top panel displays the results of a triplicate experiment with one preparation of PhK and the bottom graph is another triplicate experiment with a separate preparation of PhK. The data was fit using KaleidaGraph and the Hill equation. Individual data points in each triplicate sample are represented by closed, black circles and the average is indicated with a horizontal red line. Error bars, shown in red, represent standard deviation of the triplicate samples. Data was fit to the average, weighted with the standard deviation, and is represented by the dashed red line. (B) The same as in (A) but with non-activated PhK at 40 °C. (C) The same as in (A) but with phosphorylated PhK at 40 °C.

Table 4.1 Summary of kinetic data for non-activated and phospho-activated PhK at 30 & 40 °C.

	Temp	Specific Activity ($\mu\text{mol P/min/mg}$)	Km ($\mu\text{M GP}$)	Vmax ($\mu\text{mol P/min/mg}$)	n _H
Non-activated	30 °C	1.37 \pm 0.03 1.76 \pm 0.05	ND	ND	ND
Phospho-activated	30 °C	4.7 \pm 0.2 4.5 \pm 0.3	56 \pm 14 61 \pm 20	7.5 \pm 1.0 8.0 \pm 1.6	1.22 \pm 0.12 1.20 \pm 0.09
Non-activated	40 °C	4.9 \pm 0.3 4.2 \pm 0.4	34.6 \pm 5.3 37.4 \pm 5.9	6.34 \pm 0.54 6.53 \pm 0.63	1.33 \pm 0.06 1.28 \pm 0.07
Phospho-activated	40 °C	7.6 \pm 0.3 6.8 \pm 0.4	58 \pm 20 41 \pm 8	12.0 \pm 2.7 9.35 \pm 1.1	1.22 \pm 0.12 1.30 \pm 0.08

Two values are given for each value, indicating the results from independent experiments with different preparations of PhK.

reported here (60-fold increase vs. 2-fold increase), and is likely due to the fact that our non-activated PhK at 40 °C has a much higher basal activity than their non-activated enzyme at 30 °C.

The data from the saturation curve were fit to both the Michaelis-Menten equation and the Hill equation and the error of the calculated constants was compared to determine the equation that best fit the data. The Hill equation produced the best fit, which was somewhat unexpected because there has been no prior evidence of PhK displaying any cooperative binding of GP. PhK does have four catalytic kinase sites per hexadecamer, suggesting that a single PhK molecule could bind four molecules of GP. The number of GP molecules that bind to PhK at saturation has not been studied in great detail; however, one study suggested that two GP dimers bind to one PhK hexadecamer (116). With regard to other PhK ligands, Ca^{2+} was the only one to display any hints of cooperative binding, but those reports contradicted each other, with one reporting negative cooperativity and the other showing positive cooperativity (57, 172). Ca^{2+} binds PhK very tightly ($K_d = 17 \text{ nM}$) and low amounts of contaminating Ca^{2+} likely complicated those studies. Although the Hill numbers reported here are relatively modest ($n_H = 1.29\text{-}1.46$), these results raise the interesting possibility that one GP binding may affect the binding of subsequent GP molecules.

Does this kinetic data give us more insight into how PhK is regulated *in vivo*? Perhaps, but it is hardly the full story. In these experiments, only a small range of PhK's activity was sampled in order to avoid the problematic lag. The lag was minimized so linear initial velocity could be readily measured and fit to classic kinetic analysis, but hysteresis may be an integral part of PhK's activation *in vivo*. Enzyme hysteresis describes the slow response of an enzyme to a rapid change in substrate concentration and manifests as either a lag or burst in product formation, depending on whether the enzyme transitions to a more or less active state, respectively (173). Hysteresis may be caused by a slow conformational change, oligomerization of the protein, or dissociation of subunits induced by effector binding (174). For many hysteretic

enzymes, the cause of the hysteresis can be puzzled out by pre-incubation with effectors or substrates. PhK's lag can be equally diminished by different treatments, including auto-phosphorylation and pre-incubation with Mg^{2+}/Ca^{2+} . PhK's lag may be more likely due to a slow conformational change, based on the observation that sub-complexes of PhK (dimers of $\gamma\delta$ and trimers of $\alpha\gamma\delta$) do not display hysteresis (48). Additionally, the lag was not specific to GP phosphorylation because PhK's ATPase activity also had a lag in product formation (86). At the low intracellular pH levels in exercising muscle, PhK likely has a pronounced lag that may play a role in its regulation during muscle contraction.

The observation that PhK is more active at the physiological temperature of a rabbit has important implications for its regulation *in vivo*. PhK's basal activity was much higher at 40 °C vs. 30 °C; and although phosphorylation and allosteric effectors still stimulate activity, their effect is greatly diminished (81). Phosphorylation and allosteric effectors may therefore be more important under conditions when the intracellular pH changes and the basal activity of PhK are reduced.

Materials and Methods

Enzymes

Non-activated PhK was purified from New Zealand White rabbit psoas muscle as previously described (75), dialyzed into 50 mM HEPES (pH 6.8), 0.2 mM EDTA, and 10% sucrose (w/v), and stored at -80 °C. PhK concentration was determined spectrally at 280 nm using an absorbance coefficient of 1.24 mL $mg^{-1}cm^{-1}$ (137). GP was also isolated from New Zealand White rabbit muscle as described previously (34), and recrystallized with Mg^{2+} and AMP. After removal of AMP by dialysis into 10 mM HEPES (pH 6.8), GP was stored at -80 °C. The concentration of GP was determined spectrally at 280 nm with an absorbance coefficient of 1.30 mL $mg^{-1}cm^{-1}$ (167). The catalytic subunit of murine PKA was from New England Biolabs (Ipswich, MA). $[\gamma\text{-}^{32}P]\text{ATP}$ was purchased from PerkinElmer (Boston, MA).

PKA Phosphorylation of PhK

Quantification of subunit phosphorylation by PKA was carried out as previously described (75). The reaction mixtures contained 50 mM β GP, 50 mM Tris (pH 6.8), 1 mg/ml PhK, 0.5 mM EDTA, 0.04 μ g/mL PKA and were initiated with 10 mM $\text{Mg}(\text{CH}_3\text{CO}_2)_2$ and 0.2 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. At the indicated times aliquots were removed, quenched by dilution into reducing SDS buffer, run on SDS-PAGE, and phosphorylation quantified. Assays were performed in triplicate.

Additionally, phospho-activated PhK was generated by PKA phosphorylation for kinetic analysis. Phosphorylation was initiated by addition of MgATP into a reaction containing 20 mM β GP (pH 6.8), 0.1 mM EDTA, 0.1 mM DTT, 2 μ g/mL PKA, 550 μ g/mL PhK, 5 mM $\text{Mg}(\text{CH}_3\text{CO}_2)_2$ and 1.7 mM ATP. The reaction was run at 30 °C for 6 min before quenching with addition of EDTA to a final concentration of 10 mM. Approximately 2 mol PO_4 is incorporated into the PhK protomer.

Crosslinking

PhK (437.5 μ g/ml) was crosslinked by DFDNB (67 μ M) at 10 °C intervals between 0 and 40 °C in the presence of 50 mM HEPES (pH 6.8) and 0.1 mM EGTA. The reaction was quenched after 6 min in a reducing SDS buffer resulting in final concentrations of: 219 μ g/mL PhK, 63 mM Tris (pH 6.8), 10% glycerol, 2.5% β -mercaptoethanol, 2% SDS, and trace Coomassie. Aliquots were run on 6-18% gradient polyacrylamide gels, followed by staining with R250 Coomassie (0.1%) and Bismark Brown (0.02%) in 7% acetic acid and 40% methanol. Gels were destained in 7% acetic acid and 5% methanol. Crosslinking reactions were carried out in quintuplicate to confirm reproducibility.

Activity Assay

PhK activity was measured by the incorporation of $^{32}\text{PO}_4$ into GP by a filter paper assay (175). The reaction solution was pre-equilibrated for 2 min at the assay temperature and assays were initiated by addition of MgATP. The assays contained 20 mM HEPES, 25 mM βGP (pH 7.2), 100 mM $\text{K}(\text{CH}_3\text{CO}_2)_2$, 0.05 mM EDTA, 0.25 mM CaCl_2 , 25 mM β -mercaptoethanol, 1 mM DTT, 10 mM $\text{Mg}(\text{CH}_3\text{CO}_2)_2$, 2.5 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 6 mg/mL GP and 0.2 $\mu\text{g/mL}$ PhK. When GP concentration was varied (0.78 μM - 100 μM), the concentrations are noted in the figure legend. The molecular mass of the GP monomer (97,300 Da) was used to calculate molarity. Experiments were run in triplicate on at least two separate preparations of PhK to confirm reproducibility.

Analysis of Kinetic Data

The kinetic data were analyzed using the KaleidaGraph program to generate a weighted least-squares fit of the data. Multiple equations were applied to determine the best fit, and the Hill equation best fit the data with the smallest standard errors on the generated kinetic constants. Under all conditions, no more than 10% of the GP was phosphorylated by the completion of the assay/time point.

Conclusions and Future Directions

Phosphorylase kinase (PhK) catalyzes the phosphorylation and activation of glycogen phosphorylase (GP) as an essential step in the glycogenolysis cascade. Glycogen is present in nearly all mammalian tissues and supports a variety of biological processes, such as muscle contraction, blood glucose homeostasis, and long-term memory formation. Additionally, mutations in glycogen metabolizing enzymes, such as PhK and GP, lead to glycogen storage diseases that range in severity depending on the specific mutations and tissue afflicted. Continued investigation into the activity of PhK is therefore of great interest to better understand glycogen metabolism in both normal and pathological biology.

The discovery of PhK, which was the first protein kinase to be isolated and characterized in 1955, was a seminal event in the history of biochemistry. Although PhK has been studied for 63 years, it remains a challenging enzyme to work with, in part because of its large size. The enzyme complex has a mass of 1.3 MDa and contains 16-subunits composing four ($\alpha\beta\gamma\delta$) tetramers. Currently, we have no high resolution structures of the hexadecameric complex, but decades of structural studies using lower-resolution methods have revealed the likely arrangement of the subunits in the complex and subunit-subunit changes associated with activation. The γ subunit is the catalytic subunit and has a typical kinase domain, based on sequence similarity and crystal structures of isolated, truncated γ . The remaining three subunits, which comprise over 80% of PhK's mass, are the regulatory α , β , and δ subunits. The regulatory subunits inhibit γ in the non-activated complex and are the targets of allosteric effectors and reversible phosphorylation. A great deal of work still needs to be done to delineate the structures and regulatory mechanisms of α , β , and δ . The goal of this work was to revisit several aspects of PhK's activity and explore new and expanded roles for the enzyme's individual

subunits. The work presented here gives new insights regarding the participation of specific subunits in PhK's activation and substrate recognition.

PhK is activated by phosphorylation of the N-termini of the β subunits. The results presented in chapter II from the oxidative crosslinking of the β subunits link two important events in the activation of PhK: stimulation of the catalytic γ subunit and changes in intra- β subunit interactions. These results argue for a critical role of the N-terminus of β in inhibiting γ in the non-activated complex and mediating important structural changes upon its phosphorylation. Future work looking at the specific inhibitory interactions between β and γ would help expand our proposed model for activation. As methods to express multi-subunit complexes like PhK continue to advance, mutagenesis of β and γ may provide new ways to probe their interaction.

Prior to the work presented in chapter III, very little was known about the binding interface between PhK and its protein substrate GP. Other than the obligatory interaction between the phosphorylation site on GP and the active site on the γ subunit, there was no direct evidence for interactions between GP and any of the remaining regions of PhK. Other protein kinases are known to have docking sites that bind substrates at locations separate from the site of phosphorylation. These docking sites often vary from one protein kinase to another and can play important roles in the binding and phosphorylation of substrates. The discovery of direct interactions between GP and the regulatory α and β subunits establishes that there are substrate docking sites on PhK. The α and β subunits, along with γ , comprise the GP binding interface, but much work is left to be done to define the exact binding sites. In the future, chemical crosslinking coupled with mass spectrometry could reveal the exact residues between GP and PhK that crosslink with one another. The location of these residues may give important clues as to how these docking sites on α and β assist in substrate binding and phosphorylation.

PhK was previously found to undergo surprising activation between the standard assay temperature of 30 °C and the rabbits' physiological temperature of 40 °C. In chapter IV, evidence is presented for a temperature-dependent conformational change in the γ and β subunits between 30 and 40 °C. The observation of conformational changes in the β subunit is consistent with the model for activation of PhK presented in chapter II and suggests that conformational changes in β are a common feature of PhK activation. Several aspects of PhK's activity are changed at the higher temperature. For example, at 30 °C, non-activated PhK displays hysteretic behavior, but at 40 °C, the hysteresis is nearly abolished. Without the hysteretic behavior, which has hindered kinetic studies of PhK in the past, we studied the activation of PhK by phosphorylation at physiological temperature in terms of its kinetic parameters. Phosphorylation of PhK at 40 °C appears to coincide with an increase in V_{max} and a small increase in the K_m for GP. Additionally, the substrate saturation data were best fit to the Hill equation, suggesting that there may be some cooperativity in GP binding to PhK. For over 60 years, PhK has been studied almost exclusively at 30 °C. Given the surprising differences in the enzyme at physiological temperature, everything we have learned about PhK's structure, activity, and regulation should be re-considered in light of these findings. Undertaking these experiments is critical to understanding PhK's likely structure and behavior *in vivo*.

Appendix: Screening Kinases

Introduction

Protein kinases comprise a large family of enzymes that mediate cellular signaling processes. The human genome is estimated to contain 518 such kinases, constituting about 1.7% of all human genes (176). All protein kinases share a conserved sequence of approximately 300 residues and a highly conserved bilobal structure. Protein kinases carry out the phosphorylation of protein targets, often on Ser, Thr, or Tyr residues, but phosphorylation of additional amino acids, such as His, has also been reported (177). The phosphorylation of target sequences and the subsequent alteration of that target's structure and/or activity allows kinases to regulate diverse cellular processes, such as cell proliferation, insulin signaling, inflammatory responses, and sugar metabolism.

Efficient signal transduction requires cellular kinases to display a considerable amount of substrate selectivity. Some kinases are reported to be less selective than others, phosphorylating several proteins. For example, the α isoform of CK1 (formerly known as casein kinase) phosphorylates 14 protein targets *in vivo* and *in vitro* (178). On the other hand, phosphorylase kinase (PhK) is reported to phosphorylate only one protein *in vivo*, glycogen phosphorylase (GP). The factors governing kinase substrate specificity are not fully understood. Known substrates typically contain a sequence that matches a kinase's consensus sequence, but another protein with the same exact sequence may not be a substrate for the same kinase. This inability to identify kinase substrates based on sequence alone has important implications for researchers. At this time, there is no straightforward method to identify kinases that phosphorylate a given protein target.

There are several different approaches to screening kinases for the ability to phosphorylate a protein target; each approach has pros and cons associated with it that should be carefully considered.

1. ***In silico* prediction of kinases that phosphorylate a target protein.** Several programs exist to predict kinases that phosphorylate a given protein sequence. Using these predication programs is a fast and convenient way to identify candidate kinases that have consensus sequences within a protein of interest. *In silico* prediction of bona fide protein kinases is speculative, however, and any candidate kinases must be experimentally validated.
2. **Kinase inhibitor libraries.** Pharmacological inhibition of kinases by small molecules is a large area of research, and several kinases have relatively selective inhibitors commercially available. Phosphorylation of a target protein may be monitored in lysates or cells in the presence and absence of selective kinase inhibitors. Inhibition of substrate phosphorylation by a given inhibitor may indicate which kinase(s) target the protein of interest. This approach requires a method to reliably measure substrate phosphorylation in a lysate and likely requires additional validation with purified kinase.
3. **Co-immunoprecipitation (co-IP) coupled to mass spectrometry.** Potential binding partners of a protein of interest can be identified using co-IP and mass spectrometry. Among the proteins pulled down with the protein of interest may be a kinase that could phosphorylate the target protein. If a kinase is identified, it would still require secondary validation of both interaction and phosphorylation. Additionally, kinase-substrate interactions may be fleeting and not be captured by co-IP.
4. **Direct screening using purified kinases.** Purified kinases can be directly screened by measuring phosphorylation of a target protein *in vitro*. This is a laborious approach, but one that produces immediate results. Screening kinases with this approach takes time, careful consideration of individual kinase regulation and activity, and requires purified kinase, which can be challenging to produce and costly to purchase.

Phosphorylation of the hexadecameric PhK complex, $(\alpha\beta\gamma\delta)_4$, by cAMP-dependent protein kinase (PKA) or by PhK itself *via* autophosphorylation stimulates its activity. Non-

activated PhK purified from rabbit muscle, however, already contains several moles of phosphate in the non-activated complex (179, 180). It is not clear if this “endogenous” phosphate is incorporated *in vivo* by PKA, autophosphorylation, or some other kinase(s).

A recent study suggests that PhK exhibits a small glycosyl hydrolase activity, likely at an active site in the glucoamylase-like domain of the α subunit of PhK (66). The α subunit contains a short multiphosphorylation domain in its C-terminus, and phosphorylation of α only modestly stimulates PhK activity (104). It is therefore of interest to determine if any kinases, other than PKA or PhK, selectively phosphorylate α and if that phosphorylation stimulates PhK’s hydrolase activity.

PhK’s own substrate, GP is reported only to be phosphorylated by PhK. This notion of a single GP kinase has been held for over 60 years without being systematically tested. Additionally, the phosphorylation site at the N-terminus of GP is predicted by *in silico* methods to be targeted by several additional kinases, including PKC and PKA.

Herein, multiple protein kinases were screened for the ability to phosphorylate PhK and GP using a direct approach to measure the transfer of the γ phosphoryl group of ATP to PhK or GP by purified protein kinases or partially purified muscle lysate.

Results and Discussion

Phosphorylation of PhK by Purified Kinases

PhK was screened as a substrate for six kinases: CK1 α , GSK3 β , Cdk5, PKG1 α , Akt2, and AMPK α 2. The major challenge in the design and analysis of these experiments was the potentially high background caused by PhK autophosphorylation during the course of the experiment. Ca²⁺ is required for PhK activity (42) and was therefore excluded in the kinase screening assays to minimize autophosphorylation, especially given the high concentrations of PhK present in the assay. Despite the exclusion of Ca²⁺, Ca²⁺-independent autophosphorylation was clearly present throughout the kinase screen (Figure 5.1, open circles). The reason for the

Ca²⁺-independent autophosphorylation is not known. Although the autophosphorylation is clearly present, it is much slower (~10-fold less) than the Ca²⁺-dependent activity normally observed with PhK (data not shown). In the future, pending technical advancements in recombinant protein expression of large, multi-subunit proteins, expression of a kinase-dead PhK would allow for proper screening of novel PhK kinases without the complication of background autophosphorylation.

AMPK α 2, GSK3 β , Cdk5, and Akt2 showed no apparent phosphorylation of PhK under the conditions tested. As an example, PhK phosphorylation with and without Akt2 is shown in the bottom panel of Figure 5.1. AMPK was previously reported to not phosphorylate PhK (181), consistent with the results presented here. GSK3 β did not phosphorylate PhK, but is known to prefer substrates that already possess a phosphoserine near the target residue (182). Therefore, GSK3 β could perhaps phosphorylate PhK after an appropriate priming phosphorylation event by another kinase and thus cannot yet be eliminated as a potential PhK kinase. CK1 α does appear to cause a slight increase in the phosphorylation of PhK (Figure 5.1, top), however this increase is very modest.

PKG1 α , on the other hand, displayed relatively robust phosphorylation of PhK (Figure 5.2). Incorporation of phosphate into PhK's individual subunits was determined using SDS-PAGE to separate the PhK subunits for analysis. PKG1 α appeared to phosphorylate both α and β , similar to PKA, and no phosphorylation of the γ or δ subunits of PhK was detected. Importantly, cGMP, the essential activator for PKG1 α , did not stimulate PhK autophosphorylation, indicating that the

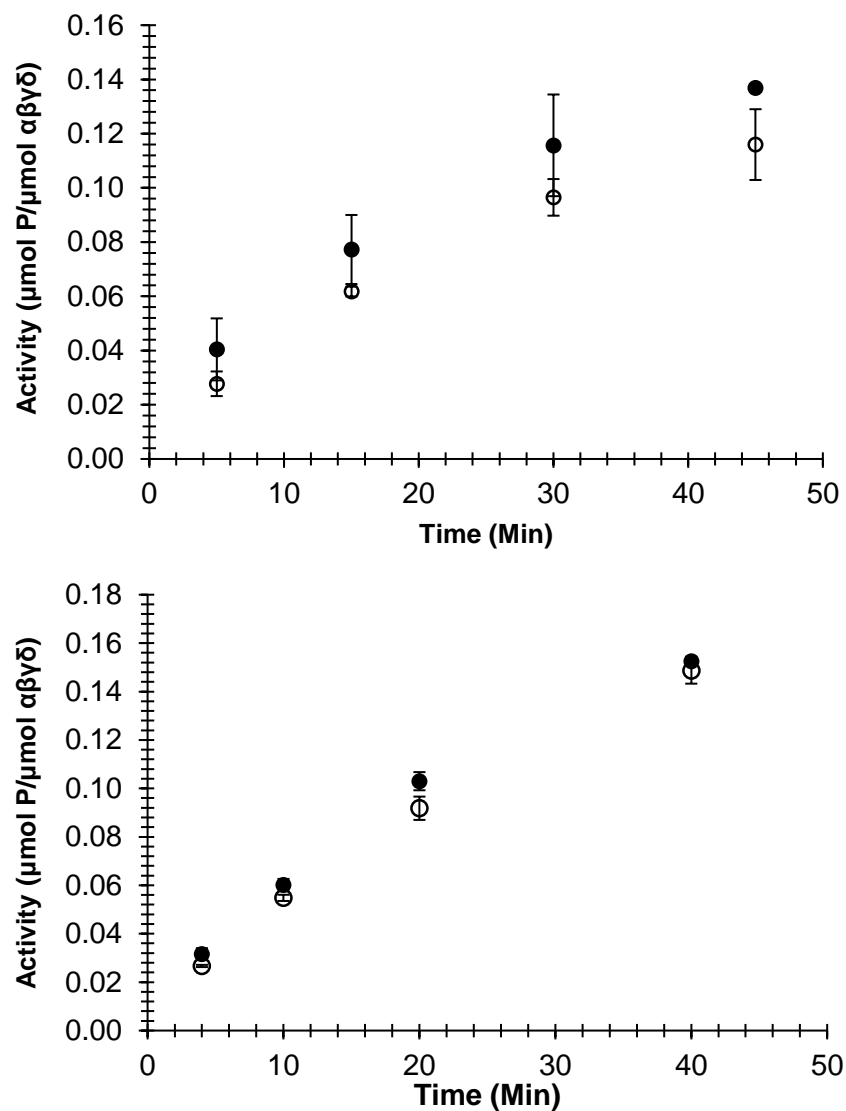


Figure 5.1 Screening PhK as a Substrate for CK1 α (top) and Akt2 (bottom). The reactions were performed as described under Materials and Methods. The closed circles are reactions containing CK1 α (top) or Akt2 (bottom). Controls without CK1 α or Akt2 were performed to determine PhK autophosphorylation (open circles). Error bars represent the standard deviation of triplicate samples.

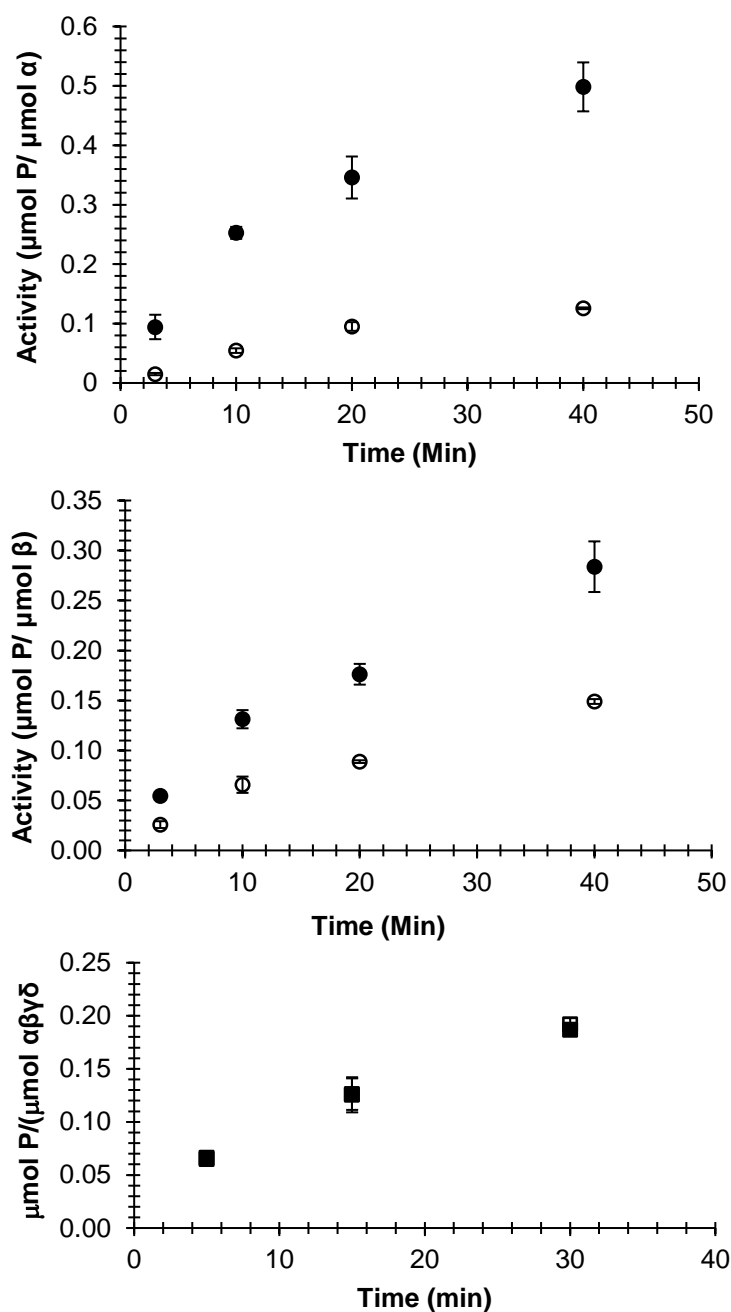


Figure 5.2 Phosphorylation of the α (top) and β (middle) subunits of PhK by PKG1 α . PhK was phosphorylated and run on an SDS-PAGE gel as described under Materials and Methods. The reaction contained 25 mM Tris/ β GP (pH 7.0), 0.5 mM EGTA, 0.1 mM DTT, 350 $\mu\text{g/mL}$ PhK, 0.1 μM cGMP, 15 $\mu\text{g/mL}$ PKG1 α , 2 mM $\text{Mg}(\text{OAc})_2$, and 0.1 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. PKG1 α phosphorylated PhK (closed circles) above the levels of autophosphorylation when PKG1 α was absent (open circles). PhK autophosphorylation was also tested in the absence (open squares) and presence (closed squares) of 1 μM cGMP and showed no difference (bottom). Error bars represent the standard deviation of triplicate samples.

increase in phosphorylation was due to PKG1 α and not cGMP-induced autophosphorylation. PhK's recently discovered glycosyl hydrolase activity was not stimulated by PKG1 α phosphorylation in a separate series of experiments.

PKG was previously identified as a PhK kinase (183) in agreement with the results presented here. Studying the influence of PKG1 α phosphorylation on PhK's kinase activity *in vitro* and *in vivo* in the future will be essential in determining the biological significance of this phosphorylation event.

Screening for PhK Kinases in Partially Purified Muscle Lysates

Partially purified muscle lysate was also screened for endogenous kinases that phosphorylate purified PhK. During the standard purification protocol, PhK is separated from the bulk of the muscle tissue lysate by acid precipitation. The large quantity of supernatant that is generally discarded contains the majority of soluble muscle proteins but lacks PhK. The supernatant was dialyzed into 20 mM HEPES (pH 7.0) to remove small molecules and PhK phosphorylation was measured in the absence and presence of 3.5 mg/mL lysate. For these experiments, high background counts from the lysate were avoided by isolating PhK's α and β subunits using SDS-PAGE. Phosphorylation of PhK was stimulated modestly by addition of the lysate (data not shown). Different effectors were added into the reaction to see if general types of kinases could be identified as potential PhK kinases. Ca²⁺ was tested as an effector and stimulated lysate phosphorylation of the α and β subunits above the level of autophosphorylation by Ca²⁺-activated PhK alone (Figure 5.3). This increase in phosphorylation may be due to a Ca²⁺-dependent kinase or activation of PhK by exogenous calmodulin (CaM), which is well-documented (184, 185). Purified PKC α , a Ca²⁺-dependent kinase, was screened as a putative PhK kinase but was not found to phosphorylate PhK.

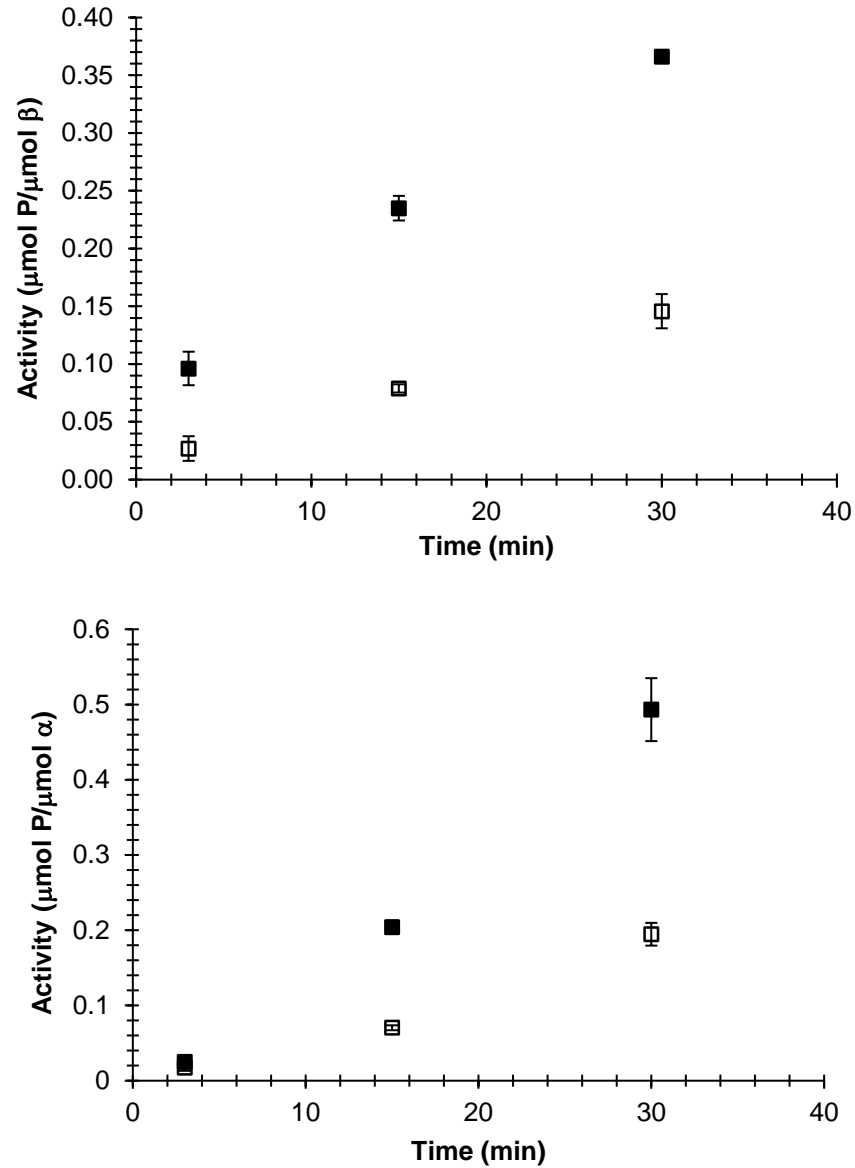


Figure 5.3 Phosphorylation of the α (top) and β (bottom) subunits of PhK by partially purified muscle lysate in the presence of Ca^{2+} . PhK was phosphorylated by 3.5 mg/mL lysate in the presence of 1 mM CaCl_2 and run on an SDS-PAGE gel as described under Materials and Methods. The error bars represent the standard deviation of triplicate samples.

It may be that PhK is phosphorylated by another isoform of PKC, another Ca^{2+} - or CaM-dependent kinase that wasn't tested here, or is simply being activated by exogenous CaM.

Phosphorylation of GP by Purified Kinases

GP was screened as a substrate for nine kinases: Akt2, GSK3 β , Cdk5, AMPK α 2, CK1 α , PKA, PKC α , PKC δ , and PKC η . GP has one known phosphorylation site at its N-terminus, Ser14, that is predicted to be solvent exposed and flexible. Computational prediction of kinases that phosphorylate Ser14 using programs such as NetPhos or GPS (186, 187) suggests that Ser14 is a potential substrate for several kinases other than PhK. In particular, PKC was particularly favored as a potential GP kinase.

From the initial screen of Akt2, GSK3 β , Cdk5, AMPK α 2, CK α , and PKC α , only PKC α appeared to phosphorylate GP, but only to a small degree (Figure 5.4). Two additional isoforms of PKC were then tested and failed to phosphorylate GP more than observed with PKC α . PhK was included in the kinase panel as a positive control for GP phosphorylation. After 10 min, the phosphorylation of GP by PKC α and PhK was similar (0.012 vs. 0.013 $\mu\text{mol P}/\mu\text{mol GP}$), but PhK far outpaces PKC α phosphorylation by 60 min (Figure 5.4, middle). PhK-phosphorylated GP catalyzes AMP-independent activity, but when GP was phosphorylated by PKC α for 10 min, no stimulation of GP's AMP-independent activity was observed (Figure 5.4, bottom). This may imply that PKC α phosphorylates an entirely different site on GP.

GP was also tested as a protein substrate for PKA. While full length GP is not typically a substrate for PKA (72, 188), a tetradecapeptide corresponding to residues 5-18 of GP, containing the phosphorylatable Ser14, is as good of a substrate for PKA as for PhK, suggesting that secondary or tertiary features of the full-length protein somehow block PKA phosphorylation (92). Previous groups have posited that GP may become a substrate for PKA in the presence of GP effectors

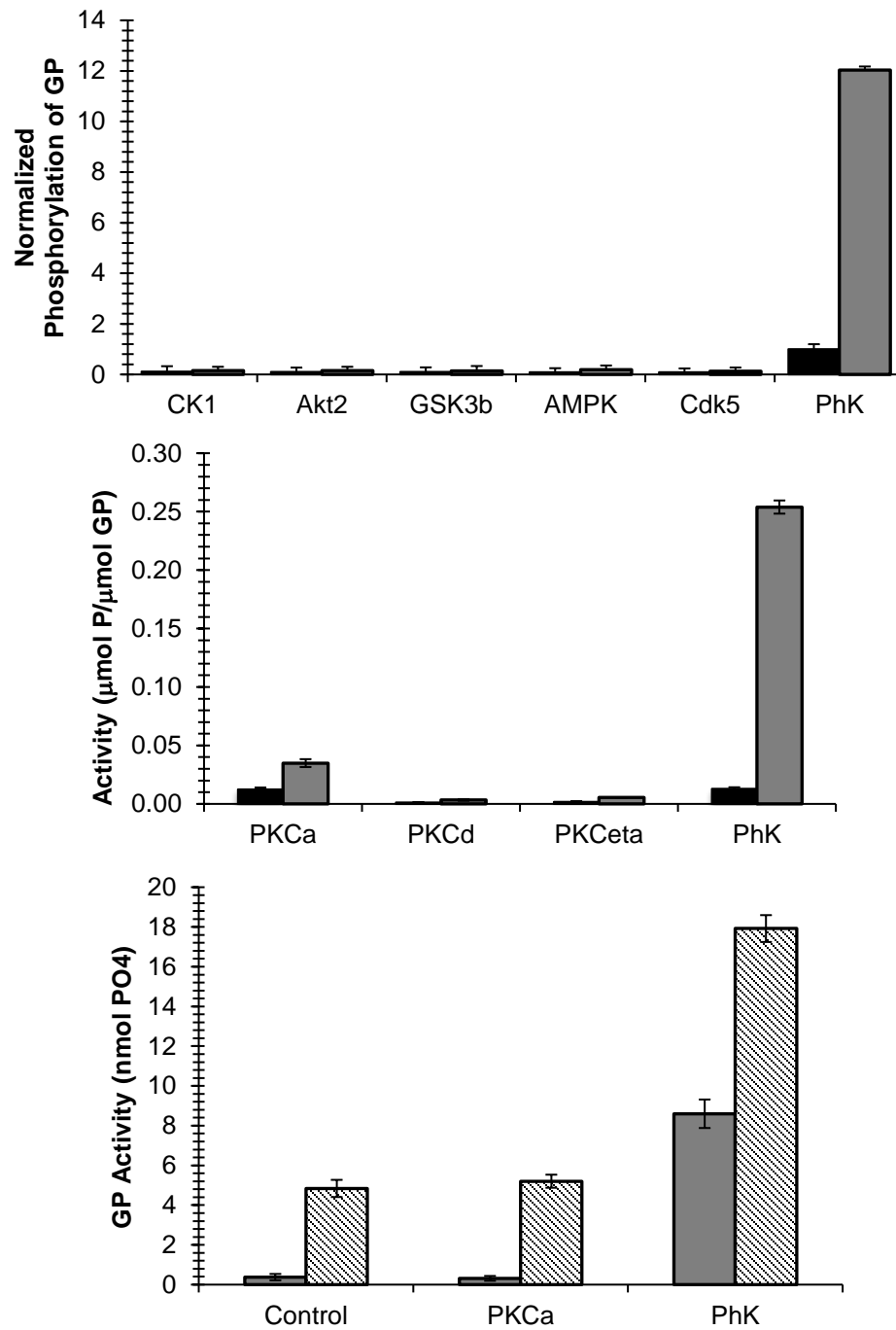


Figure 5.4 Screening kinases for the ability to phosphorylate GP. GP was screened (top and middle) as a substrate for CK1 α , Akt2, GSK3 β , AMPK α 2, Cdk5, PKC α , PKC δ , and PKC η as described under Materials and Methods. PhK was included as a positive control for GP phosphorylation. Bottom, GP's AMP-dependent (dashed bars) and AMP-independent (gray bars) activities measured after phosphorylation by PKC α or PhK for 10 min. The phosphorylation reaction was quenched with EDTA (final concentration 10 mM) and GP was diluted in cold 20 mM MOPS (pH 6.8) before being assayed as described under Materials and Methods. Control GP was not phosphorylated for comparison. Error bars represent the standard deviation of triplicate samples.

known to alter GP's conformation (19, 72). Tessmer *et al.* (72) tested PKA phosphorylation of GP in the presence of multiple GP effectors and salts. Under no conditions tested did PKA phosphorylate GP or apo-GP, which lacks its essential PLP group. Recently, however, Biorn *et al.* (93) demonstrated that PKA could slowly phosphorylate a mutant GP with a single Ala mutation at Arg16, two residues C-terminal (P+2) to the phosphorylation site. Although an Arg at the P+2 position is favored by PhK and disfavored by PKA (189, 190), the fact that a single residue change could permit PKA phosphorylation was intriguing. It seemed reasonable therefore to revisit the potential phosphorylation of wild-type GP by PKA in the presence of GP effectors, including some previously tested (AMP, glucose-6-P, and glycogen (72)) as well as some never-before tested (inosine monophosphate (IMP), uridine diphosphate glucose (UDPG), and glucose). In the presence of near-saturating amounts of these GP effectors, GP was not phosphorylated by PKA under any conditions tested (data not shown). In agreement with earlier studies, PKA cannot phosphorylate Ser14 on GP, despite its ability to phosphorylate a corresponding tetradecapeptide. This further supports the hypothetical existence of a structural feature on full-length GP that sterically blocks or otherwise impairs phosphorylation of its N-terminus by PKA.

Materials and Methods

The kinases included in the screen were: His₆-tagged PKG1 α from EMD Millipore, GST tagged CK1 α from Abcam, GST-tagged GSK3 β from Abcam, His₆-tagged AMPK α 2 from Abcam, His₆-tagged Cdk5:p35 from Invitrogen, AKT2 from Abcam, PKC α from Abcam, the catalytic subunit of PKA from New England Biolabs, and GST-tagged PKC α , δ , and η from SignalChem.

Non-activated PhK was purified from New Zealand White rabbit psoas muscle as previously described (75), dialyzed into 50 mM HEPES (pH 6.8), 0.2 mM EDTA, and 10%

sucrose (w/v), and stored at -80 °C. GP was also isolated from New Zealand White rabbit muscle as described previously (34) and recrystallized with Mg^{2+} and AMP.

The PKC activation mixture containing lipids and Ca^{2+} was purchased from SignalChem. The remaining reagents were purchased through Sigma Aldrich or Fisher Scientific.

Phosphorylation of PhK

Most kinase assays were performed using a radioactive filter paper assay (175). PhK autophosphorylation in the absence of additional kinases was monitored as a control in all reactions. In all experiments, assays were initiated with the addition of MgATP and performed at 30 °C. For CK1 α , PKC α , Akt2, and PKG1 α the reaction mixture contained 25-50 mM Tris/ β GP (pH 7.0-7.5), 0.1 mM EGTA, 0.1 mM DTT, 300-350 μ g/mL PhK, 1-4 μ g/mL kinase, 1-2 mM $Mg(OAc)_2$, and 0.1 mM [γ - 32 P]ATP. The reactions containing PKC α also contained 0.15 mM $CaCl_2$, 0.1 mM 1,2-diacyl-sn-glycero-3-phospho-L serine, and 0.01 mM 1,2-dioleoyl-sn-glycerol, and reactions containing PKG1 α contained 0.1 μ M cGMP. Samples were collected from the reactions up to 45 min post-initiation.

AMPK α 2, GSK3 β , and Cdk5 assays were also initiated with the addition of MgATP, and the final concentrations were 40 mM BGP (pH 7.0), 0.5 mM EDTA, 50 mM NaF, 0.1 mM DTT, 1.5 mM $Mg(OAc)_2$, 0.25 mM [γ - 32 P]ATP, 500 μ g/mL PhK, and 1 μ g/mL kinase. For AMPK α 2 reactions, 0.25 mM AMP was also included. Reactions were carried out at 30 °C for 30 min.

When possible, positive phosphorylation controls were performed with known protein substrates to demonstrate that the kinases were active under the conditions tested. Such controls were performed with PKC α , Cdk5, and CK1 α and they were found to robustly phosphorylate their known substrates.

The supernatant from the acid precipitation step in the PhK purification protocol was dialyzed into 20 mM HEPES (pH 7.0) and used as a source of endogenous muscle kinases. The total protein concentration of the partially purified lysate was determined by a BCA assay,

and phosphorylation of PhK was measured in the absence and presence of the lysate. The reactions were initiated by addition of MgATP and contained 20 mM Tris/βGP (pH 6.8), 0.5 mM EGTA, 50 mM NaF, 0.1 mM DTT, 350 μg/mL PhK, 4 mM Mg(OAc)₂, 3.5 mg/mL lysate, and 1 mM [γ -³²P]ATP. When included, the concentration of CaCl₂ was 1 mM. Reactions were carried out at 30 °C for up to 30 min.

Phosphate incorporation into the α and β subunits of PhK by PKG1 α or partially purified muscle lysate was performed by quenching the reactions in SDS buffer and running the samples on a 6-18% SDS-PAGE gel as described previously (124). After running the samples on a 6–18% gels, staining, and destaining, the α and β bands were excised, decolorized in 350 μL 30% H₂O₂ at 104 °C for 1 h, and diluted in 4 mL scintillation fluid.

For the initial screen, only one preparation of PhK was tested as a substrate. When phosphorylation was detected (*i.e.*, PKG1 α phosphorylation of PhK) the experiment was repeated with a separate preparation of PhK to confirm reproducibility. Deviations from any of the methods described above are indicated in the figure legends.

Phosphorylation of GP

GP was tested as a substrate for nine kinases: AMPK α 2, Akt2, GSK3 β , CK1 α , Cdk5, PKA, PKC α , PKC δ , and PKC η . Reactions were initiated with addition of MgATP and the final reaction mixture contained 20 mM HEPES (pH 7.4), 0.1 mM EDTA, 0.1 mM DTT, 5 mg/mL GP, 5 μg/mL kinase, 10 mM Mg(OAc)₂, and 1 mM [γ -³²P]ATP. When PKC isoforms were tested, a PKC activator cocktail containing Ca²⁺ and lipids was included. When AMPK α 2 was tested, 1.5 mM AMP was included. The reaction proceeded at 30 °C and samples were collected at 10 and 60 min to measure phosphate incorporation.

Positive phosphorylation controls were performed for PKC α , PKC δ , and PKC η with histone as the protein substrate and demonstrated that these kinases were active under the conditions tested.

GP was also tested as a substrate for PKA in the presence of different GP effectors. The reactions were initiated with addition of MgATP and included 20 mM Tris (pH 7.4), 0.1 mM EDTA, 5 μ g/mL PKA, 0.5 mg/mL GP, 10 mM Mg(OAc) $_2$, and 0.5 mM [γ - 32 P]ATP. When added to the reaction individually, the final concentrations of GP effectors were 2 mg/mL glycogen, 1 mM AMP, 25 mM UDPG, 7 mM IMP, 0.1 mM glucose-6-P, and 8.5 mM glucose. These concentrations are 10-fold higher than the reported K_i/K_a for these GP effectors. The reactions proceeded for 10 min at 30 °C.

GP Activity

GP was assayed in the direction of glycogen synthesis by quantifying phosphate cleaved from glucose-1-P. The assay was initiated with addition of GP and the final reaction solution contained 20 mM MOPS (pH 6.8), 2.5 mM glucose-1-P, 0.1 mg/mL glycogen, and 10 μ g/mL GP. When present, the concentration of AMP was 1 mM. The glycogen, purchased from Sigma, was further purified by ethanol precipitation and dialysis to remove contaminating nucleotides and phosphate. GP was assayed for 5 min at 30 °C and 20 μ L aliquots were quenched by addition of 1 mL of freshly prepared malachite green reagent (0.12 mM malachite green, 2.25 mM ammonium molybdate, 830 mM H $_2$ SO $_4$, 0.02% PVA). The malachite green reaction was allowed to develop for 10 or 12 min and the absorbance at A630 was measured. The color development was continuous, so the A630 measurements were carefully timed. A standard phosphate curve was generated for each experiment and was linear between 0 and 20 nmol PO $_4$.

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